PATENT
0825-0166P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant:	Henrik GAROFF et al.	Conf.:	8395
Appl. No.:	09/901,106	Group:	1636
Filed:	July 10, 2001	Examiner:	GUZO, DAVID
For:	DNA EXPRESSION SYSTEMS BASED ON ALPHAVIRUS		

DECLARATION UNDER 37 C.F.R. 1.132

Commissioner for Patents
P.O. Box 1450

Sir:

I, Peter Liljeström, hereby declare as follows:

1. I have been awarded degrees of B. Sc. (1977), M. Sc. (summa cum laude 1979), Ph.lic. (summa cum laude 1981) and Ph.D. (summa cum laude 1986) awarded from the University of Helsinki. I presently hold a position as Professor at the Microbiology and Tumor Biology Center (MTC) at the Karolinska Institute, Sweden. I also hold a position as Head of the Department of Vaccine Research at the Swedish Institute for Infectious Disease Control, Sweden.

2. The Examiner takes a position that the following phrases in the claims of the '106 application are not described in the specification and therefore the present claims of the '106 applications are not entitled to the filing date of the corresponding International Application:

a. helper cells;

b. limitations of more than one helper RNA; and

c. characteristics of the helper RNAs.

3. I understand from explanation by a U.S. Patent attorney that the legal requirement for adequate written description is met if the specification describes the claimed invention in sufficient detail that one of ordinary skill in the art can reasonably conclude that the inventor had possession of the claimed invention. Possession of the invention is shown by description of the claimed invention with all of its limitations using words, structures, figures, diagrams and formulas that fully set forth the claimed invention. Review of this issue by the Examiner is to be made from the standpoint of one of skill in the art at the time the application was filed and should include a determination of the field of the invention and the level of skill and knowledge in the art.

4. The present specification certainly provides written description of an alphavirus expression system utilizing a defective alphavirus vector ("recombinant alphavirus vector") that is supported by complementation of one or more defects in the alphavirus by expression of genes resident in one helper vector. To date, this is not at all disputed by the Examiner.

5. The present specification provides written description of modularity of possible helper functions in several respects. First, at page 29, lines 1-2 the specification indicates that "independent overlapping subclones" were used to assemble a complete SFV genome for the vector pSP6-SFV4. The maps at Figures 4A and 4B show what subclones were used and also present a restriction map showing what restriction sites can be used to separate the various structural protein transcripts. This map, together with drawing Figure 2 suggests that at least the capsid protein and the E2-6k-E1 proteins can be made on separable transcripts.

Furthermore, these proteins are made in a non-overlapping sequence and so it would have been understood by the molecular biologist of ordinary skill at the time the application was filed that the structural gene for each of these proteins could be placed under a separate transcriptional promoter and be

individually expressed. This is supported by results from our laboratory published prior to the priority date of the present application which definitely show that one can divide the structural gene region of an alphavirus and express the proteins separately. The relevant publications are provided attached as Exhibits A to C, respectively:

Garoff, H., Huylebroeck, D., Robinson, A., Tillman, U. and Liljeström, P. (1990, September issue). "The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation." J. Cell Biol. 111:867-876. (Exhibit A)

Garoff, H., Liljeström, P., Metsikkö, K., Lobigs, M. and Wahlberg, J. (1990). "Formation and function of the Semliki Forest virus membrane." In New Aspects of Positive-Strand RNA Viruses, Eds Brinton, M.A. and Heinz, F.X., Chapter 24, pp 166-172. (Exhibit B)

Liljeström, P. and Garoff, H. (1991, submitted July 9, 1990/Accepted October 1990). "Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor." J.Virol.65:147-154. (Exhibit C)

The results in Exhibit A demonstrate that the p62 protein can be independently expressed from its own plasmid and yet be directed to the cell membrane. Furthermore, its signal sequence directing insertion of the protein into the membrane can be used to insert a heterologous protein into the membrane. Exhibit C shows that co-translation of the p62 and 6K-E1 proteins is not necessary for proper insertion of these proteins into the cell membrane, since deletion of most of the 6K protein from the polyprotein still allows independent insertion of the E1 protein into the membrane from a signal sequence independent of the p62 signal sequence. In Exhibit B, the Examiner might take note of the text at page 168, column 2, lines 4, ff. that the p62 and E1 proteins had been expressed "from separate coding units of engineered cDNA sequences" and yet can associate in the host cell membrane.

Furthermore, at page 32, line 5 of the specification, there is disclosure that:

> The system allows SFV variants defective in structural protein functions, or recombinant RNAs derived from the expression vector construct obtained in Example 2, to be packaged into infectious virus particles. Thus, this system allows recombinant RNAs to be introduced into cells by normal infection . ... As is shown in Fig. 9, after transcription in vitro of pSFV1-recombinant and helper cDNAs, helper RNA is cotransfected with the pSFV1 - recombinant derivative, the helper construct providing the structural proteins needed to assemble new virus particles, and the recombinant providing the nonstructural proteins

needed for RNA replication, SFV particles comprising recombinant genomes being produced.

Page 9, lines 11-24 of the specification, describes generally the idea that a helper vector is to complement the lack of structural proteins encoded in the replicon vector.

6. At the time the application was filed, the level of ordinary skill in the art of defective virus vector/helper vector expression systems was such that several groups had developed viral expression systems using a plurality of helper vectors to perform complementation of defective viral vector functions.

These systems were developed principally to enhance the safety of such systems for therapeutic uses to a level over and above that achieved by making the vector replication incompetent. It was known that use of a plurality of helpers would help reduce the likelihood of formation of recombinant, replication competent vectors during the packaging process. Simplisticly, the greater number of recombination events needed to create a replication proficient virus the less likely it would be for this phenomenon to occur.

Attached as Exhibits D to G are copies of papers from the scientific literature, written prior to the December 1990 priority date of the present application, showing these facts.

Exhibit D, J.P. Morgenstern et al., Nucl. *Acids Res.* 18:3587-3596 (June 1990) describes a Moloney murine leukemia virus packaging system that utilizes a defective replicon vector pBabe that is packaged using a cell line, omega E that expresses the gag-pol protein and an ecotropic env protein from separate plasmids.

Exhibit E, D. Markowitz et al., *Ann. Rev. NY Acad. Sci.* 612:407-414 (1990), describes another retroviral system using a packaging cell line in which the viral gag and pol genes are resident on one plasmid and env genes are resident on a second plasmid.

Exhibit F, A.D. Miller, Hum. *Gene Ther.* 1:5-14 (Spring 1990) is a review article that describes several viral gene expression systems that utilize a plurality of helper vectors within a single cell line.

Exhibit G, A.K. Pattnaik et al., Proc. Natl. Acad. Sci. USA 88:1379-1383 (1991), accepted for publication in November 1990, describes a system based on the Vesicular Stomatitis Virus in which five different plasmids, each directing expression of a particular

VSV protein, were used to support assembly of defective VSV viruses.

7. That the development of a safe and effective viral vector system for gene expression in a therapeutic setting is an object of the present invention is set forth, for example, at page 6, lines 31-34 (described as a problem in the prior art to be solved).

8. The specification of the present application describes that the structural protein genes of alphaviruses can be separately expressed. From this description, together with the fact that others at the time the application was filed were making viral vector-based gene expression systems with a plurality of helper vectors, one of ordinary skill in the art would reasonably conclude that I, together with my co-inventor Dr. Garoff, had in our possession an invention residing in an alphavirus expression system utilizing "at least one separate helper RNA encoding the structural protein(s)" and/or "at least a first and second helper RNAs separate from said replicon and separate from each other" at the time the present application was filed.

9. Furthermore, the specification clearly describes at least two kinds of mutations in the spike proteins E2 and E3. The specification also describes in a general fashion that the spike proteins are responsible for the activity of entry of the virus into a cell and that mutation of these proteins results in a lack of infectivity. See, e.g. page 10, lines 14-24, page 18, lines 28-32 and page 19, lines 7-8. There is explicit description of a conditional lethal mutation in the E3 protein provided in Example 5 of the specification. There is explicit description of mutation of E2 by inserting a short oligonucleotide in Example 4. Thus, the specification clearly contemplates a range of kinds of mutations and that mutation of one or more of the E1, E2 or E3 spike proteins is a suitable way to accomplish a non-replicative form of an alphavirus vector. That is, mutation or deletion of the spike proteins from the alphavirus vector is suggested as a way to create a replication defective vector, which can be assembled into an infectious particle by use of one or more helper vectors or helper cells that provide the complementing normal spike proteins.

10. Still further, at page 10, lines 29 to 34, the specification states, "The RNA molecule of the present invention is derived by in vivo or in vitro transcription of a cDNA clone,

originally produced from an alphavirus RNA and comprising an inserted exogenous DNA fragment encoding a desired genetic trait. Also, at page 12, lines 4-8, the specification states that, "The present invention is also related to a method to produce transformed animal host cells comprising transfection of the cells with the present RNA molecule or with the present transcription vector comprised of cDNA and carrying an exogenous DNA fragment." Thus, one of ordinary skill in the art at the time the present application was filed would understand that I and Dr. Garoff had in our possession an invention encompassing animal host cells, including "helper cells", that had been transformed with RNAs described in the specification or with cDNAs corresponding to such RNAs.

11. The above explanation plainly establishes that one of ordinary skill in the art, reading the present specification, would understand that the concepts of helper cells, use of a plurality of helper RNAs (or corresponding cDNAs), and mutation or deletion of the spike proteins from the alphavirus vector as a way to create a replication defective vector, which can be assembled into an infectious particle by use of one or more helper vectors or helper cells that provide the complementing normal spike proteins, were subject matter in the possession of

the inventors named on the present application when the present application was filed.

12. I hereby declare that all statements made herein of my own knowledge are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated this 25 day of January, 2005.

Peter Liljeström

The Signal Sequence of the p62 Protein of Semliki Forest Virus Is Involved in Initiation but Not in Completing Chain Translocation

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Abstract. So far it has been demonstrated that the signal sequence of proteins which are made at the ER functions both at the level of protein targeting to the ER and in initiation of chain translocation across the ER membrane. However, its possible role in completing the process of chain transfer (see Singer, S. J., P. A. Maher, and M. P. Yaffe. *Proc. Natl. Acad. Sci. USA.* 1987. 84:1015–1019) has remained elusive. In this work we show that the p62 protein of Semliki Forest virus contains an uncleaved signal sequence at its NH_2-terminus and that this becomes glycosylated early during synthesis and translocation of the p62 polypeptide. As the glycosylation of the signal sequence most likely occurs after its release from the ER membrane our results suggest that this region has no role in completing the transfer process.

BIOSYNTHESIS of proteins at the ER can be subdivided into several steps. These are (*a*) targeting of translation complexes to the ER membrane; (*b*) synthesis and transfer (translocation) of the polypeptide chain across the lipid bilayer; and (*c*) protein maturation in the lumen of ER (chain folding, disulphide bridge formation, glycosylation, and oligomerization). The mechanisms for these processes have been studied extensively during recent years (Kornfeld and Kornfeld, 1985; Wickner and Lodish, 1985; Rapoport, 1986; Lodish, 1988; Rothman, 1989). A most important finding has been that all proteins made at the ER carry a signal sequence (also called signal peptide), a hydrophobic peptide which is usually located at the NH_2-terminal region of the polypeptide chain. One function of the signal peptide is to achieve targeting of the polysome to the ER membrane (Rapoport, 1986). When the signal sequence emerges from the ribosome it binds to the signal recognition particle, which mediates binding of the polysome to the docking protein in the ER. After this another function of the signal sequence is expressed, that is to interact with some components of the ER membrane and thereby initiate translocation of the polypeptide chain into the lumen of the ER (Gilmore and Blobel, 1985; Robinson et al., 1987; Wiedmann et al., 1987). Further synthesis of the polypeptide then continues with concomitant chain translocation. An important but as yet unresolved question is whether the signal sequence has any role in the translocation process per se or whether its functions are limited to the targeting and translocation-initiation steps. For instance, Singer and co-workers (1987*a*) have suggested a translocator protein model in which the signal sequence helps to keep the machinery open for chain transfer.

It is specifically this last question we have addressed in the present work. We describe the characteristics and behavior of the uncleaved signal sequence of the p62 protein of Semliki Forest virus (SFV)[1] upon translocation across the ER membrane in vitro. The p62 protein is one subunit of the heterodimeric spike protein of the SFV membrane (reviewed in Garoff et al., 1982). It is made as a precursor protein together with the other structural proteins of SFV, i.e., the nucleocapsid protein, C, and the other spike subunit, E1. The three proteins are synthesized from a 4.1-kb long mRNA in the order C, p62, and E1, and separated by cleavage of the growing precursor chain. During synthesis of the p62 polypeptide at the ER all but a 31 residue COOH-terminal portion and the membrane anchor is translocated across the membrane. The p62 signal sequence has so far been only roughly localized to the NH_2-terminal third of the polypeptide chain (Garoff et al., 1978; Bonatti et al., 1984). We show here that the signal sequence of p62 consists of a 16 residue peptide at its NH_2-terminal region. This region includes one out of four glycosylation sites (Asn_{13}) for N-linked oligosaccharide on the p62 chain. We also demonstrate that the glycosylation of the p62 signal sequence occurs early during chain translocation. As this modification of the signal region most likely correlates with its release into the lumen of ER it follows that the signal sequence of p62 is probably only needed for an initial step in chain translocation and not to

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1. *Abbreviations used in this paper*: ATA, aurintricarboxylic acid; SFV, Semliki Forest virus.

maintain the competence of the chain translocation machinery throughout p62 chain transfer.

Materials and Methods

Materials

T4 Polynucleotide kinase, SP6 Polymerase, and most restriction endonucleases were obtained from Boehringer Mannheim Biochemicals (Mannheim, FRG), except for Xmn I and Sac I (New England Biolabs, Beverly, MA). T4 DNA Polymerase was purchased from Bethesda Research Laboratories (Rockville, MD), RNase inhibitor from Promega Biotech (Madison, WI), proteinase K from E. Merck (Darmstadt, FRG), and Xho I linker (dCCTCGAGG) from Collaborative Research (Lexington, MA). T4 DNA ligase was a gift from W. Fiers (Laboratory of Molecular Biology, State University of Ghent, Belgium) and purified from an *E. coli* strain that thermo-inducibly overproduces this enzyme from a bacterial expression vector (Remaut et al., 1983). Ribonucleotides (from equine muscle) and spermidine used for RNA synthesis, together with PMSF, diethyl-pyrocarbonate (DEPC), and BSA (fraction V) were from Sigma Chemical Co. (St. Louis, MO). The cap analogue $m^7G(5')ppp(5')G$ and protein A-Sepharose CL-4B were from Pharmacia (Freiburg, FRG). RNase inhibitor was from Promega Biotech. Methylated, ^{14}C-labeled proteins used as molecular mass markers and Enhance (for fluorography) were from DuPont de Nemours (NEN Research Products, Dreieich, FRG). Reticulocyte lysate was prepared from rabbits according to established procedures (Jackson and Hunt, 1983). Some lysate of the same kind (N.150) was also purchased from Amersham International (Amersham, UK) together with stabilized L-[^{35}S]methionine (SJ.1515). Dog pancreas rough microsomes (50 A_{280}/ml) were partly provided by B. Dobberstein (European Molecular Biology Laboratory) and partly prepared by ourselves according to Kaderbhai and Austen (1984). The rabbit antimouse dhfr antibody was a generous gift from E. Hurt (European Molecular Biology Laboratory). DNA fragments were purified in LMP agarose (Bethesda Research Laboratories). The acceptor peptide N-benzoyl-Asn-Leu-Thr-N-methylamide and the allothreonine (aThr) containing nonacceptor peptide N-benzoyl-Asn-Leu-aThr-N-methylamide were synthesized according to Erickson and Merrifield (1976; see also Tillmann et al., 1987). Aurintricarboxylic acid (ATA) was from Sigma Chemical Co.

General DNA Methods

Small scale plasmid DNA preparations were done using the alkali-SDS method essentially as described by Birnboim and Doly (1979). Large quantities of plasmids to be used for in vitro transcription were prepared by lysozyme-Triton lysis of the bacteria, followed by CsCl-EtBr banding (Kahn et al., 1979). EtBr was removed by several extractions with isopropanol and, after fivefold dilution, the DNA was precipitated twice with ethanol and further purified over a Biorad A-50m column. Restriction endonucleases and DNA-modifying enzymes were used according to the suppliers instructions. Removal of the 3' sticky end from the Sac I site in pGEM2-alphaG (Zerial et al., 1986) with T4 DNA Polymerase was done at 15°C (2 h), dNTPs were added (end concentration 100 μM each), and the DNA was subsequently filled in at 15°C for 1 h. All ligations were done at 24°C for 4 h except for linker ligations (4°C, 16 h). All other molecular biological manipulations were done using slightly modified standard protocols (Maniatis et al., 1982).

In Vitro Transcription and Translation

In vitro transcription (0.3 μg supercoiled template DNA per 10 μl vol) in the presence of SP6 RNA Polymerase (6–8 U) and the cap structure was carried out as previously described (Zerial et al., 1986). In vitro translation reactions using a rabbit reticulocyte lysate were performed at 30°C essentially as described (Melancon and Garoff, 1986). 1.5 μl of the in vitro synthesized RNA was translated in a total volume of 15 μl. Potassium, magnesium, and spermidine concentrations were 100, 1.2, and 0.375 mM, respectively. When indicated, 1 μl of ER membranes was included. In some translocations the membranes were pretreated with 200 μM peptide for 5 min on ice. The final peptide concentration in the total translation mixture here was, after addition of the pretreated membranes, adjusted to 100 μM. To obtain partial synchronization of translation, ATA was added after a preincubation of 1.5–3.0 min (Borgese et al., 1974). A final ATA concentration of 0.075 mM was found to be sufficient for inhibiting initiation of chain synthesis (see control in Fig. 6, lane *l*). Higher concentrations of ATA inhibited first translocation and then also chain elongation. For protease protection experiments, proteinase K was added to a final concentration of 0.1 mg/ml and the samples were incubated at 0°C for 30 min in the presence or absence of 1% Triton X-100. Proteolysis was stopped by the addition of PMSF (final concentration 2 mg/ml) and samples were kept at 0°C for 5 min before further processing for electrophoresis (Cutler and Garoff, 1986). Bands containing labeled protein were visualized by fluorography. Quantitation of proteins was done by cutting the bands out of the dried gel, solubilizing them with Protosol (from DuPont de Nemours, NEN) according to the instructions of the manufacturer, and finally counting the ^{35}S radioactivity in a liquid scintillator (Wallac LKB, Turku, Finland). The localization of the bands on the dried gel was done with the aid of the fluorograph in transillumination.

Alkaline Treatment of Cell-free Translation Mixtures

15-μl translation mixtures were adjusted to pH 11–11.5 by adding an appropriate volume (pretitrated) of 0.1 N NaOH. After a 10-min incubation on ice the samples were separated into a pellet fraction and a supernatant fraction by centrifugation through a 100-μl alkaline sucrose cushion (Gilmore and Blobel, 1985) for 10 min at 30 psi in an airfuge (Beckman Instruments, Inc., Palo Alto, CA) using the A-100/30 rotor and cellulose propionate tubes precoated with BSA (1% solution). The entire supernatant was removed, neutralized with 1 N HCl, diluted 2.5 times with water, and then precipitated by adding 3.5 vol of acetone. These precipitated proteins and pelleted membranes (obtained from the airfuge tube) were taken up in 4% SDS by incubating at 56°C for 15 min and then processed for immunoprecipitation reactions as described below.

Immunoprecipitation of Fusion Protein from Reticulocyte Lysate

Total translation mixtures were adjusted to 4% SDS, then boiled for 4 min and diluted 1:2 with water. 4 vol of immunoprecipitation buffer (2.5% Triton X-100, 190 mM NaCl, 60 mM Tris-HCl, pH 7.4, 6 mM EDTA, and 20 μg PMSF/ml) and 2 μl of antibody were added for 16 h at 4°C. The mixture was briefly centrifuged (2–3 min in an Eppendorf minifuge) and to the supernatant one fifth volume of a 1:1 slurry of protein A beads were added and incubated at 24°C for 2 h under constant agitation. The beads were collected and washed four times with 1 ml RIPA buffer (Gielkens et al., 1976) by centrifugation, followed by a single wash with a buffer containing 150 mM NaCl, 10 mM Tris-HCl pH 7.4, and 20 μg PMSF/ml. The beads were then taken up in excess gel loading buffer (Cutler and Garoff, 1986), heated at 95°C for 5 min, and cleared by centrifugation before loading the immunoprecipitate on the gel.

Plasmid Constructions

Constructions of pGEM2alphaGX and pGEM2dhfrX. For the construction of the final fusion protein-coding plasmids used in this study we first had to make plasmids pGEM2alphaGX, which are derived from pGEM2alphaG and pGEM2dhfrX, which are derived from pGEM2dhfr (Zerial et al., 1986). Plasmid pGEM2alphaG contains a 548 bp-long Nco I–Pst I fragment encompassing the entire chimpanzee alpha-globin coding region between the Hinc II and Pst I sites of the polylinker of the plasmid pGEM1 (Promega Biotech). The Nco I site contains the translation initiation codon from alpha-globin (Zerial et al., 1986). An Xho I site, allowing subsequent in-frame ligations of SFV sequences, had to be introduced in pGEM2alphaG. Therefore, this plasmid was cut (upstream of the Nco I site) with Sac I, the 3' sticky ends removed with T4 DNA Polymerase, an Xho I octamer linker introduced and, after cutting with Xho I, the plasmid was religated at low DNA concentration (1 μg/ml). Plasmid pGEM2alphaGX then contains the 2,057 bp-long Xho I–Pvu I fragment needed for the construction of the fusion protein-coding plasmid pC62alphaG.

An intermediate construct, analogous to pGEM2alphaGX, and also containing a unique Xho I site, was needed for the constructions of dhfr-containing plasmids. For this purpose we inserted the Xho I linker into partially Xmn I cut pGEM2dhfr (Zerial et al., 1986). After cutting the linkers, linear plasmid was purified on agarose gel and religated. Since the second Xmn I site in pGEM2dhfr is located in the beta-lactamase coding region of the vector (Sutcliffe, 1979) and insertion of an Xho I site by an octamer linker will result in an ampicillin-sensitive *E. coli* phenotype after transformation, only the desired pGEM2dhfrX construct was obtained. From this plasmid, an Xho I-Pvu' I fragment of at least 2,012 bp (the precise length

of the cDNA insert, i.e., the length of the 3' untranslated region of dhfr, is not known in pGEM2dhfr) was used for the construction of pC62dhfr.

Construction of the Fusion Protein-coding Plasmids pC62alphaG and pC62dhfr. Plasmid pGEM1-SFV (also called pG-SFV-15/5; Melancon and Garoff, 1986) contains a reengineered cDNA copy of the SFV 26S mRNA sequences cloned as a Bam HI fragment in the Bam HI site of the polylinker downstream of the SP6 promoter in the plasmid pGEM1 (Promega Biotech). From the SFV plasmid, a 2,381 bp-long Pvu I-Xho I fragment, containing the coding sequences for the capsid protein and the NH_2-terminal region of the p62 protein, was isolated. The Xho I–Pvu I fragments from pGEM1-SFV, pGEM2alphaGX and pGEM2dhfrX were isolated and ligated at a 1:1 molar ratio to obtain pC62alphaG and pC62dhfr, respectively. Plasmid DNAs from ampicillin-resistant colonies were screened and compared to the starting vectors by restriction analysis.

Altogether, the SFV-alpha-globin cDNA fusion results in a complete C region and 40 codons from the 5' end of the p62 region fused to the whole of the alpha-globin coding sequence (see Fig. 1). Eight new codons have been introduced at the point of cDNA fusion. In the SFV-dhfr construction the C region and the 40 first codons of p62 are fused to the dhfr coding sequence such that one new codon is introduced and the first 31 codons of dhfr are lost.

Construction of Plasmid p62dhfr. For engineering of a p62 protein signal sequence-dhfr fusion protein which is not derived from a C protein-containing precursor we synthesized the whole p62 signal sequence region. Two overlapping oligonucleotides were made (DNA-synthesizer; Applied Biosystems, Foster City, CA): (1) 5' ATACACAGAATTCAGCACCATGTCCGCCCCGCTGATTACTGCCATGTGTGTCCTTGCCAATGCTACCTTCCCGTGCTTCCAGCCCCCGTGTGTACCTTGC, (2) 5' GTTATCCTCGAGCATCCGTAGTGTGGCCTCTGCGTTGTTTTCATAGCAGCAAGGTACACACGGGGGCTGGAAGCACGGGAAGGTAGCATTGGCAAGGAC. They correspond to both strands of the p62 signal sequence region of the SFV cDNA. Together they span the coding region of amino acid residues 1-40 of p62. Oligo 1 (the coding strand) includes, in addition, the region coding for initiator methionine of the C protein plus its 5' flanking sequences (5' AGCACCATG). At the extreme 5' end of this oligo we have added the recognition sequence for Eco RI and its flanking sequences from the 5' end of the structural part of the SFV cDNA (5' ATACACAGATTC). Oligo 2 ends at its 3' end with the Xho I site which follows the signal sequence region on the p62 gene.

The two oligonucleotides were hybridized (51 complementary bases), filled in using Sequenase (United States Biochemical Co., Cleveland, OH) and restricted with Eco RI and Xho I. The resulting DNA fragment was then purified and inserted into pCp62dhfr instead of the C and p62 sequences. For this purpose the pCp62dhfr plasmid was Eco RI and Xho I restricted and the plasmid part with the dhfr sequences isolated. The resulting plasmid p62dhfr contains thus the coding sequences for the initiator methionine of C and the first 40 residues of the p62 protein, including the signal sequence, in front of the dhfr gene (see Fig. 1).

Construction of pGEM SFV d-4. This plasmid was constructed by ligating three fragments together. The first one was the major part of pGEM1, cut just after the promoter region with Hind III and Bam HI. The second fragment (Hind III–Xho I) was isolated from the plasmid pSVS-SFV (Kondor-Koch et al., 1983). This fragment contains the sequences encoding the capsid and the NH_2-terminal part of the p62 protein of SFV. The third fragment was obtained by cleaving plasmid pL1 SFV d-4 (see below) with Xho I and Bam HI and isolating the fragment containing the 3' part of the coding sequence for the p62 protein. However, it should be noted that in the d-4 version there is an exchange of 15 codons at the 3' end of the p62 gene for six aberrant ones. The corresponding p62 protein variant is called p62 d-4 (see Fig. 1). It should also be mentioned that pL1 SFV d-4 has been derived from pL1 SFV d-9, (Cutler and Garoff, 1986) by exchanging the Xho I-Cla I region containing the 3' part of the p62 coding region with the similar fragment from pSV2 SFV d-4. This latter plasmid is described in Garoff et al. (1983).

Results

Localization of the Signal Sequence of p62

To define the p62 signal sequence we have studied the translocation phenotype of two reporter molecules, the rabbit alpha-globin and the mouse dihydrofolate reductase (dhfr), both of which have been extended at their NH_2-termini with an NH_2-terminal 40 residue peptide from p62. The hybrid molecules were tested in a microsome-supplemented in vitro translation system. The alpha-globin and the dhfr have earlier been shown to be translocation incompetent if not extended with a heterologous signal sequence at their NH_2-termini (Zerial et al., 1986).

We first tested the expression of in vitro-made RNA from the construction pCp62dhfr in an in vitro translation system. This would be expected to yield free C protein and p62-reporter hybrid (p62-dhfr) through C-catalyzed autoproteolytic cleavage of the nascent C-p62-reporter precursor (Fig. 1) (Aliperti and Schlesinger, 1978; Hahn et al., 1985; Melancon and Garoff, 1987). Furthermore, the p62-reporter hybrid should be translocated across microsomal membranes and possibly glycosylated at Asn_{13} of the p62 sequence if the 40 residues long NH_2-terminal p62 peptide carries a signal sequence. Fig. 2 presents an SDS-PAGE

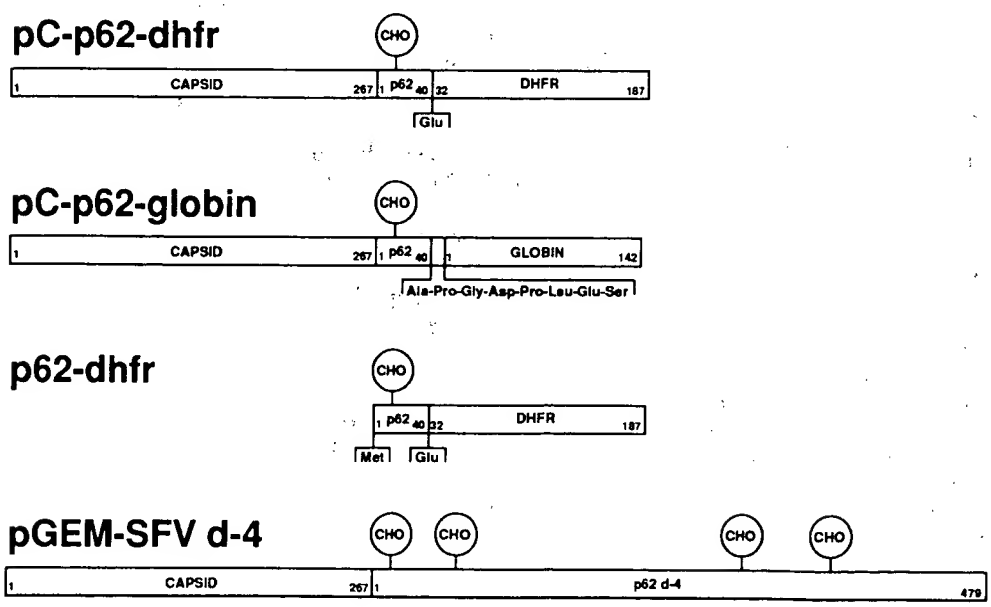

Figure 1. Composition of polypeptides coded for by plasmid pCp62dhfr, pCp62-globin, p62dhfr, and pGEM SFV d-4. Numbers refer to amino acid residues as deduced from the known cDNA sequence of chimpanzee alpha-globin (Liebhaber and Begley, 1983), mouse dhfr (Chang et al., 1978; Nunberg et al., 1980; Crouse et al., 1982), and SFV (Garoff et al., 1982). Oligosaccharide (*CHO*) is shown to be linked to Asn residue 13 in the p62 part (Garoff et al., 1982). Additional amino acids resulting from in frame translation of the multicloning region of pGEM2 and the added Xho I linker as well as the initiator Met of p62dhfr are also indicated.

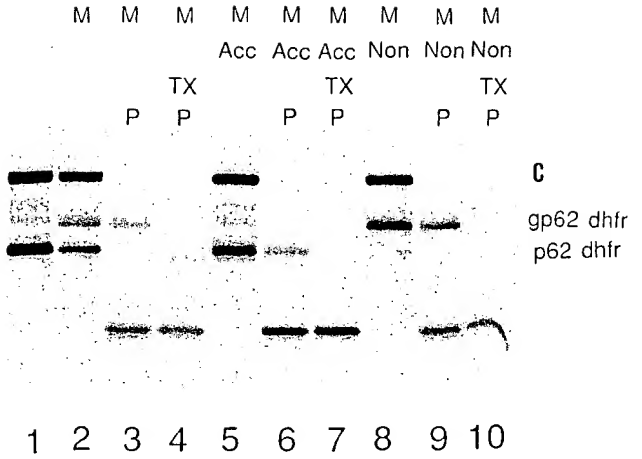

Figure 2. Translation in vitro of p62-dhfr. Plasmid pCp62dhfr was transcribed into RNA and this was used for in vitro translations in the presence (*M*) or absence of membranes. The samples were analyzed by SDS-PAGE (10%), followed by autoradiography. When indicated, an acceptor peptide (*Acc*) for N-linked glycosylation or a nonacceptor peptide (*Non*) was included in the translation. P and TX codes are used when in vitro translations have been treated with protease (*P*) in the presence (*TX*) or absence of Triton X-100. Bands corresponding to the C, the p62-dhfr, and glycosylated p62-dhfr proteins are indicated.

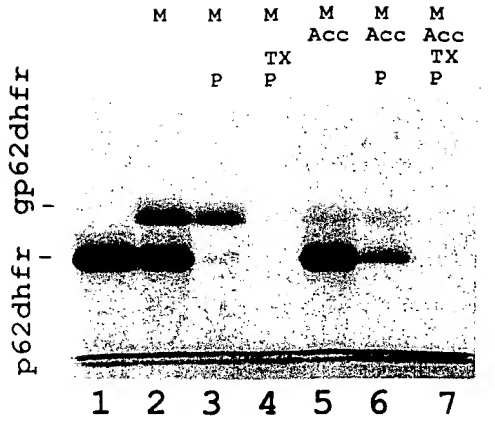

Figure 4. Translation in vitro of p62-dhfr. Plasmid p62dhfr was transcribed into RNA and this was used for translation as described in the legend to Fig. 2.

analysis showing the translocation activity of the p62-dhfr protein. In the absence of membranes (lane *1*) two major protein species were translated from the SP6-directed transcript. One of these had the expected size of C (33 kD) and the other one that of the p62-dhfr hybrid molecule (21 kD). The coding region has apparently been translated faithfully and the precursor protein cleaved efficiently. The identity of the p62-dhfr was directly proven by immunoprecipitation with a dhfr specific antiserum (see Fig. 3). The two weaker bands migrating faster than the capsid in Fig. 2, lane *1* were most likely derived from C coding sequences because they are found in all protein analyses of in vitro transcription/translation mixtures involving cDNAs with C regions (compare Fig. 6).

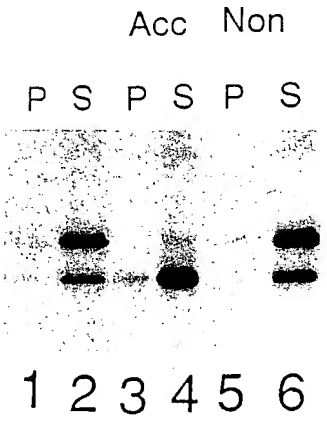

Figure 3. Immunological identification of the p62-dhfr hybrid protein and analysis of its association with membranes. RNA transcribed from pC62-dhfr was translated in vitro in the presence of membranes which in some cases had been treated with an acceptor (*Acc*) or nonacceptor (*Non*) peptide. The samples were treated, after translation, at pH 11–11.5, and the proteins then separated into a membrane-bound pellet fraction (*P*) and a supernatant fraction (*S*) by centrifugation. In all samples the p62-dhfr polypeptides were isolated using an anti-dhfr antibody. The proteins were then analyzed by SDS-PAGE (10%) and subsequent autoradiography. The slower migrating band corresponds to glycosylated and the faster one to nonglycosylated forms of p62-dhfr (compare Fig. 3).

When microsomes were added to the C-p62-dhfr in vitro translation system a new band appeared which migrated somewhat slower than the p62-dhfr band seen in the analysis of the mixture lacking membranes (Fig. 2, lane *2*). It almost comigrated with one of the two weak C derived bands. The new band apparently corresponds to p62-dhfr hybrids that have been translocated into the lumen of the added microsomes and have become glycosylated. The immunoprecipitation analysis shown in Fig. 3 confirmed the identity of this material. The protease digestions in the absence (Fig. 2, lane *3*) and presence of Triton X-100 (lane *4*) clearly demonstrated that the slower migrating p62-dhfr molecules were indeed translocated. About half of this material remains protected in the presence of intact microsomes whereas all is digested when the membranes are solubilized with detergent. In contrast, the other translated material did not show such a pronounced membrane-dependent protease resistance. Note that protease treatment of all samples yielded a resistant protein of a small size. This most likely represents a protease-resistant C fragment.

The glycosylation of the translocated p62-hybrid and its effect on the apparent size of the protein was shown in an experiment where a short peptide (Asn-Leu-Thr), which competes for N-linked glycosylation, was included during translation. Apparently only unglycosylated faster migrating p62-dhfr hybrids were formed in these conditions although chain translocation took place conferring protease resistance (Fig. 2, lanes *5–7*). Additional analyses (lanes *8–10*) illustrate that a control peptide (Asn-Leu-aThr) which cannot serve as an acceptor site for N-linked glycosylation, had no effect on the glycosylation of the p62-reporter hybrids when tested in an analogous way.

Similar studies as with pCp62dhfr were also performed with the pCp62globin coded proteins in vitro. The results (not shown) were analogous to those described above for the pCp62dhfr construct. C protein and p62-globin hybrid were synthesized in the absence of membranes. When membranes were added, a protease-protected form of the hybrid appeared. This hybrid was also glycosylated as deduced from

an experiment involving the acceptor peptide for glycosylation.

Fig. 3 (lanes *1–6*) shows the results of analyses in which we have tested whether the p62 signal sequence region confers stable membrane attachment to the p62-dhfr hybrid. Microsome-supplemented translations were adjusted to pH 11–11.5 with NaOH, incubated on ice for 10 min, and then separated into a membrane pellet and supernatant fraction by ultracentrifugation. In all samples the p62-dhfr polypeptides were isolated using an anti-dhfr antibody. SDS-PAGE shows that the hybrid protein segregates almost quantitatively into the supernatant fraction (compare lane *1* with lane *2*). In similar conditions an integral membrane protein, the human transferrin receptor, was found to sediment with the membranes into the pellet fraction and a secretory protein, Ig light chain, was only recovered in the supernatant (not shown). If the acceptor peptide for glycosylation was included in the in vitro translation and the mixture then analyzed we found that the now unglycosylated but still translocated p62-hdfr hybrids were again mostly found in the supernatant fraction (lanes *3* and *4*). Lanes *5* and *6* show the analyses with the control peptide.

To see whether the C protein exerts an influence on the translation phenotype of the p62-dhfr protein the p62dhfr plasmid (see Fig. 1), lacking the C gene, was tested. The results shown in Fig. 4 show clearly that the p62-dhfr hybrid is translocated and glycosylated in the same way as when expressed from pCp62dhfr. Thus, apart from providing a free NH_2-terminal end to the p62-dhfr protein by autoproteolysis of the C-p62-dhfr precursor the C protein has no role in the translocation process.

We conclude that the 40 residue peptide from the p62 NH_2-terminal region confers a translocation positive phenotype to the p62-globin and p62-dhfr polypeptides and therefore must contain a functional signal sequence. The translocated fusion proteins were also shown to be glycosylated. This must involve Asn_{13} of the p62 peptide as it is part of the only potential glycosylation site on the hybrid polypeptides (Garoff et al., 1980; references on dhfr sequence in legend to Fig. 1). Finally, we can also conclude that the p62 signal sequence does not provide a stable membrane anchor to the translocated chain.

Time Point of Asn_{13} Glycosylation during Polypeptide Chain Synthesis and Translocation

To define at what time point during p62-dhfr chain synthesis the Asn_{13} becomes glycosylated we performed a time-course experiment essentially as described by Rothman and Lodish (1977) (Fig. 5). In this experiment a 150-μl translation was initiated. After 1.5 min ATA was added (0.075 mM) to block additional starting of chain synthesis. Then, at 0.5-min intervals, two 7.5 μl aliquots were removed; one for mixing with 40 μl of hot PAGE sample buffer (2% SDS) and the other one for further incubation after mixing with 0.75 μl of 20% TX-100. The first sample from each time point was used for the determination of the time needed for chain completion, which is a function of the translation rate, and the other one allowed determination of the time course of glycosylation of the translocated chain. Triton X-100 solubilizes the microsomal membranes and thereby inactivates glycosylation (but not chain elongation). Therefore, only those p62-dhfr chains that have presented Asn_{13} to the glycosylation machinery before TX-100 addition have had the possibility to become glycosylated. In Fig. 5, lanes *1–10*, one can see that completed p62dhfr chains (197 residues with initiator Met) appear after a 3-min incubation from the time point of ATA addition. If one assumes constant chain initiation during the

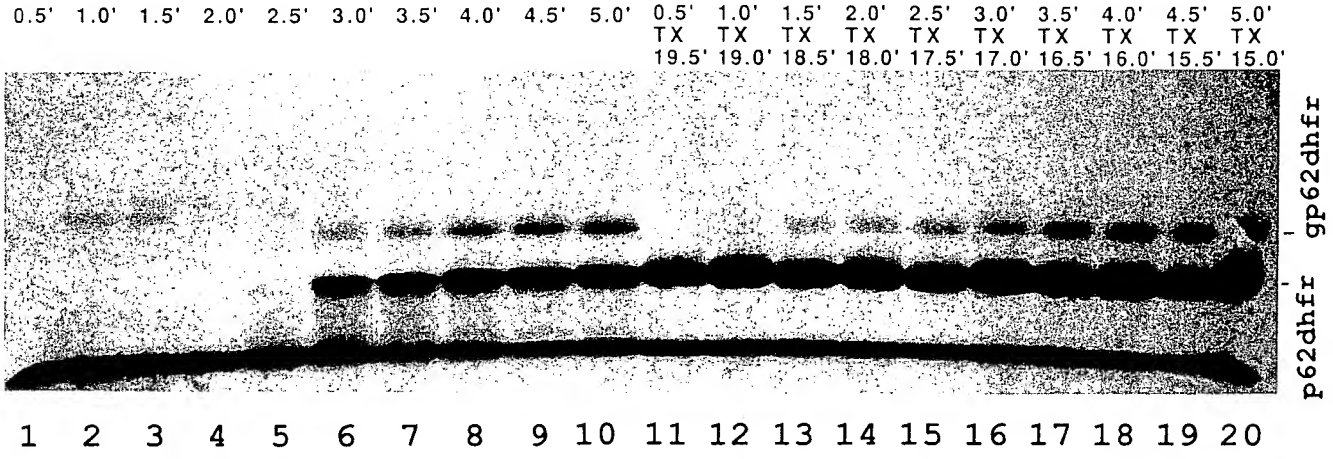

Figure 5. Time course of p62 dhfr glycosylation. A 150-μl translation was initiated. After a preincubation time of 1.5 min ATA was added to inhibit further initiation of chain synthesis. Then, at intervals of 0.5 min (indicated by 0.5', 1.0', 1.5', 2.0', 2.5', 3.0', 3.5', 4.0', 4.5', and 5.0') two 7.5-μl samples were removed, one for mixing with PAGE sample buffer and another one for mixing with TX-100 (final concentration 1%) and further incubation at 30°C (for a total time of 20 min after ATA addition as indicated by the lower row of time points in the figure). Lanes *1–10* show the samples removed for mixing with the PAGE sample buffer. From these results the approximate rate of translation can be derived. Completed chains appear in the 3-min sample. Lanes *11–20* show the samples in which the membranes have been solubilized with Triton X-100 for inactivation of the glycosylation machinery. From these analyses it is possible to estimate when Asn_{13} is modified during p62-dhfr synthesis. The first glycosylated forms are clearly visible in the 1.5-min sample, a time point where only about half of the p62-dhfr chain has been synthesized. The nature of the material in the two weak bands seen in lanes *1–5* is unclear. Their transient appearance before the completion of the p62-dhfr chain suggests that they represent complexes of nascent p62-dhfr chains.

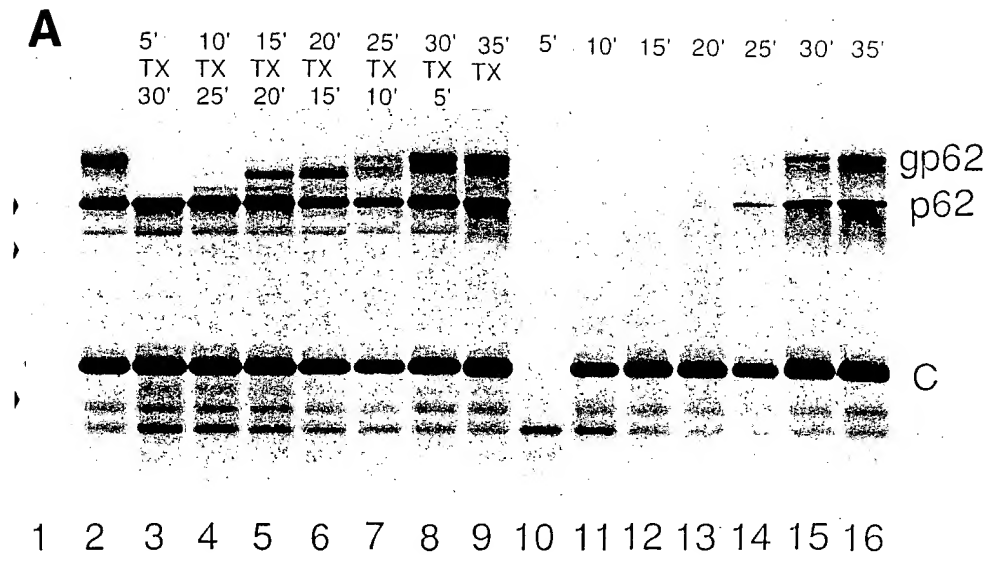

Figure 6. Time course of p62 d-4 glycosylation. Seven translations in the presence of microsomal membranes were started in parallel. After a 3-min initial incubation at 30°C, ATA was added in order to inhibit further initiation of chain synthesis. Incubation was then continued for 35 min. At the indicated time points (5, 10, 15, 20, 25, 30, and 35 min) TX-100 (*TX*) was added to stop further chain glycosylation. At the same time one half of each sample was removed and put on ice in order to measure the extent of chain elongation at each time point. All samples were analyzed by SDS-PAGE (10%) and autoradiography. Lanes *3–9* show the analysis of the samples incubated with TX-100 and lanes *10–16* the analysis of the portions put on ice at the different time points. The complete sequence of treatments for each sample is indicated by the labeling in each lane (upper row of time points indicate TX-100 addition and cooling on ice, respectively; lower row of timepoints indicate incubation in the presence of TX-100). Lanes *1* and *2* represent controls. In the experiment shown in lane *1*, ATA was added before starting a 40-min membrane-supplemented translation. In the experiment shown in lane *2* a translation with membranes was allowed to proceed for 40 min. ATA was added as in the time course samples but TX-100 was omitted. The C protein, the unglycosylated (*p62*) and the glycosylated (*gp62*) forms of p62 d-4 are labeled at right in the figure. Arrowheads at left indicate (from above) the migration of the 53-kD IgG heavy chain, the 46-kD ovalbumin, and the 30-kD carbonic anhydrase. Note that somewhat different amounts of translation mixtures have been analyzed in the various lanes (compare intensities of C and C-derived bands).

1.5-min preincubation without ATA then the total time for chain synthesis is ~3.75 min (3 + 0.75 min). This corresponds to a mean translation rate of 52.5 peptide bonds per min. Lanes *11–20* show that glycosylated chains appear in all those samples that have had the membranes intact for 1.5 min or more after ATA addition. This means that p62dhfr chains that have been elongated for ~2.25 min (1.5 min incubation after and 0.75 min before ATA addition), to the length of ~118 residues already carry a sugar unit at Asn_{13}. As ~60 residues of the nascent chain are required to span the ribosome and the lipid bilayer we conclude that glycosylation occurs when the first 50–60 residues of p62-dhfr appear within the lumen of the ER (Malkin and Rich, 1967; Blobel and Sabatini, 1970; Bergman and Kuehl, 1977; Smith et al., 1978; Glabe et al., 1980; Randall, 1983).

We also studied the timing of the glycosylation of Asn_{13} in its normal background, i.e., during p62 chain synthesis. For this experiment we used the pGEM SFVd-4 construct. This encodes the C and the p62 membrane protein variant, p62 d-4, in which a few residues of the cytoplasmic protein domain have been exchanged as compared to the wild type sequence (see Materials and Methods and Fig. 1). Fig. 6, lane *2*, shows that RNA, which has been transcribed from this construct, directs the synthesis of C and p62 d-4 chains. The protein has catalyzed correct C-p62 cleavage and the p62 signal sequence has catalyzed the insertion of about half of the p62 d-4 chains across the added microsomal membranes. These migrate as glycosylated 60–58 kD proteins in contrast to the noninserted molecules which have an apparent molecular mass of ~52 kD. The glycosylated and translocated nature of the 60–58 kD material was clearly demonstrated in experiments similar to those described above for the p62-dhfr hybrid molecule (not shown).

Altogether there are four glycosylation sites within the p62 d-4 sequence. These correspond to Asn residues at positions 13, 60, 266, and 328 (see Fig. 1). Fig. 6 (lanes *3–16*) shows the time course of the four glycosylation events during C-p62 d-4 translation. A slightly different protocol was followed in this experiment as compared to that with p62-dhfr. Seven translations were initiated in parallel and after a 3-min incubation these were put on ice and ATA was added. Elongation of the already initiated chains was then continued for a total of 35 min, however, so that Triton X-100 was added to individual samples at 5, 10, 15, 20, 25, 30, and 35 min. At these time points half of each sample was also removed and translation stopped by cooling on ice. Lanes *3–9* show the SDS-PAGE of the samples that had received Triton X-100 at different time points. We found the sequential appearance of p62 d-4 polypeptides with no carbohydrate (lane *3*), with one and two units added (seen as two new bands with slower migration in lanes *4* and *5*), with three units (lane *6*), and all four sugar units (lanes *7*, *8*, and *9*) attached to the protein backbone as the translation proceeded coordinately with time. Note that the four glycosylation events result in different degrees of increase of the size of p62 d-4. The second event causes the largest increase and the third one the smallest. As the sugar unit added at each step should be the same we think that these differences reflect some conformational changes in the p62 folding which occur coordinately with glycosylation.

In lanes *10–16* we have analyzed the samples that were withdrawn at the different times but were kept on ice. As expected, we see a sequential appearance of first the capsid

protein (in the 10-min sample) and then the p62 d-4 protein (barely visible in the 20-min sample). The p62 d-4 protein is partly present in its glycosylated and partly in its unglycosylated form. Using 21.5 min as a rough estimate for the translation time of the 746 residue long C-p62 d-4 chain (time point of p62 d-4 detection, 20 min, plus half of the 3-min preincubation time without ATA) we have calculated the translation rate and derived the approximate earliest time points when the four glycosylation sites of p62 d-4 should be available for modification. According to these, Asn_{13} and Asn_{60} should be the only sites available for glycosylation in the 10-min sample, shown in lane *4*, and the most abundant ones presented for modification in the 15-min sample, shown in lane *5*. Therefore, it appears reasonable to assume that those chains of these two samples which have obtained two sugar units carry these on the aforementioned two sites. Thus, the peptide region with Asn_{13} seems to be target for rapid modification also when present in its normal background, that is with the p62 protein.

Discussion

The fact that the 40 residue fragment of the NH_2-terminal region of the p62 protein is able to translocate two different reporter molecules into microsomes constitutes in our mind convincing evidence for signal sequence activity in this protein fragment. A more precise location of the p62 signal sequence within the 40 residue p62 fragment can be done with the aid of the known consensus features of a signal sequence. The most typical characteristic of a signal sequence is a stretch of 10–12 uncharged residues, mostly hydrophobic ones (von Heijne, 1985). This part of the signal sequence probably forms an alpha helix in the ER membrane (Emr and Silhavy, 1983; Briggs et al., 1985, 1986; Kendall et al., 1986; Batenburg et al., 1988). The only possible candidate region within the 40 residue p62 fragment having these features is the 13 residue segment between Pro 3 and Pro 17 (see box in uppermost sequence in Fig. 7). The Pro-rich region in the middle of the 40 residue fragment would not form an alpha-helix, and the COOH-terminal part of the p62 segment contains a high number of charged residues. As shown in Fig. 7 these features are conserved in all those alphaviruses where the p62 protein has been sequenced. Thus, we find the experimental results, together with the structural considerations discussed above, highly indicative that the 16 NH_2-terminal residues of p62 constitute its signal sequence.

Eventually, the signal sequence of the p62 protein becomes translocated across the membrane of the ER into its lumenal space. In here it is found as a glycosylated peptide which is part of a 66 amino acid residues long "pro"-piece of the p62 protein. This pro-peptide, called E3, is cleaved at a late stage during virus assembly (de Curtis and Simons, 1988) and is then either released into the extracellular medium as a soluble protein (Sinbis virus) or remains as a peripheral protein subunit on the virus spike (SFV) (Garoff et al., 1982; Mayne et al., 1984). Our present tests of the p62-globin and p62 dhfr hybrids in the high pH wash assay of membrane supplemented in vitro mixtures also support the notion that the p62 signal sequence does not remain bound to the membrane where it has exerted its function as a translocation signal.

In this work we like to use the glycosylation event at Asn_{13} of the signal sequence to mark the time point when the latter becomes released into the lumen of the ER. The crucial question then becomes whether it is reasonable to assume that the signal peptide has to be released from the ER membrane before it can become glycosylated. To answer this question we have to consider what is known about the topology of glycosylation as well as the way by which the p62 signal might interact with the ER membrane.

Today there is no exact information about how a signal sequence might be inserted into the ER membrane when exerting its function in chain translocation. However, the typical cytoplasmic orientation of the NH_2-termini of membrane protein chains carrying a combined signal sequence-anchoring peptide suggests that signal sequences in general might direct their function in translocation through the insertion of their hydrophobic and uncharged stretch of amino acid residues into the membrane in such an orientation that the NH_2-terminus of the signal remains on the outside of the ER mem-

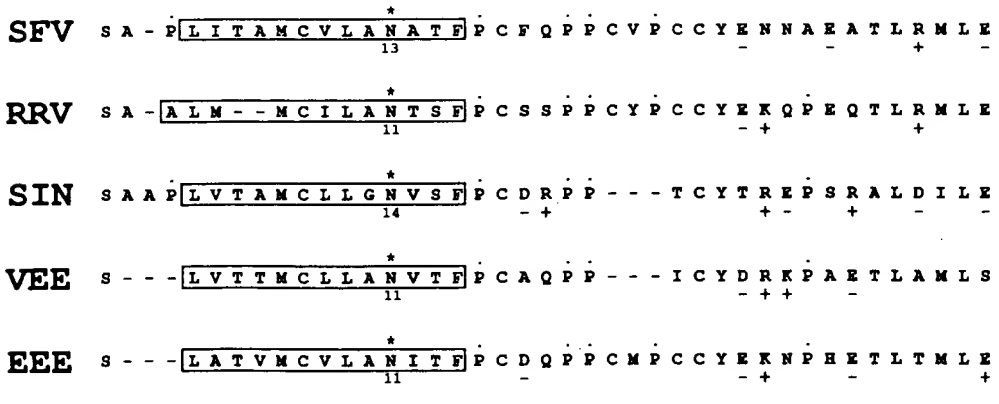

Figure 7. NH_2-terminal amino acid sequences of p62 proteins of alpha viruses. The amino acid sequences of the 40 residue NH_2-terminal peptide of the p62 protein of SFV (upper sequence) and the corresponding sequences of the p62 proteins of Ross River virus (*RRV*); Sindbis virus (*Sin*); Venezuelan Equine Encephalitis virus (*VEE*); and Eastern Equine Encephalitis virus (*EEE*) (Garoff et al., 1980; Rice and Strauss, 1981; Dalgarno et al., 1983; Kinney et al., 1986; Chang and Trent, 1987). Amino acid residues are given using the one letter code and they are numbered from the NH_2- towards the COOH-terminus. The boxes indicate that region in each sequence which best fulfills the consensus features of a signal sequence (the uncharged and hydrophobic region). The * symbols represent attachment sites for oligosaccharide and the (+) and (−) the presence of a charged amino acid side chain. Proline residues are labeled with a dot. The sequences are aligned according to maximum amino acid sequence homology.

brane (Bos et al., 1984; Lipp and Dobberstein, 1986; Spiess and Lodish, 1986; Zerial et al., 1986; see also Shaw et al., 1988). In addition, it is known from physical studies using synthetic signal peptides and artificial lipid membranes that the signal peptides readily insert into the membrane and there obtain an alpha-helical conformation (Briggs et al., 1985, 1986; Batenburg et al., 1988; Cornell et al., 1989). If the p62 signal sequence adapts such an orientation and conformation in the ER membrane it would mean that the glycosylation site at Asn_{13} would locate inside the membrane (von Heijne, 1985). In this location the site can hardly be accessible for the glycosylation machinery. (Note that in the related Ross River virus, the Venezuelan Equine Encephalitis virus and Eastern Equine Encephalitis virus the corresponding glycosylation site is even closer towards the NH_2-terminus, that is at Asn 11, see Fig. 7.) According to several recent studies, glycosylation requires the exposure of the glycosylation site in the lumen of ER. Firstly, it has been shown that the binding protein for the glycosylation site of N-linked oligosaccharides is a lumenal 57-kD protein of the ER (Geetha-Habib et al., 1988). Secondly, one study with the asialoglycoprotein receptor and another one with the Corona virus E1 membrane protein demonstrate that lumenally oriented glycosylation sites are not used on transmembrane polypeptides if they locate very close to the membrane-binding segments of the chains (Mayer et al., 1988; Wessels and Spiess, 1988). In the case of the asialoglycoprotein receptor a site was not used if located 12 residues apart from the membrane anchor, however, if moved 8 more residues apart from the anchor it became glycosylated. In the case of the Corona virus protein a site just adjacent to the combined signal sequence-anchor peptide remained unglycosylated, whereas an engineered site 24 residues further away was used for glycosylation. Such restrictions in glycosylation are most likely to be explained by sterical problems in attaching the very spacious sugar unit (Lee et al., 1984; see also Wier and Edidin, 1988) onto acceptor sites that are fixed in a position which is close to the membrane plane.

Therefore, we assume that the p62 signal sequence, with its glycosylation site at Asn_{13}, cannot become glycosylated before it has been released into ER lumen. As this glycosylation event was shown to occur at an early stage of chain translocation it follows that this signal sequence can only interact with the ER membrane during the beginning of chain translocation. In other words, the signal sequence of p62 can only function at the initiation stage of chain translocation and has no role in completing this transfer process. If the latter would be true we would have expected that the signal sequence glycosylation would have occurred first after all of the lumenal domain of the p62 d-4 chain would have been translated and translocated.

The importance of our results in this work lies in the fact that they rule out translocation models in which the signal sequence would have a role throughout the whole process of chain translocation. For instance, if the translocation site is represented by a multisubunit protein complex forming an aqueous channel across the membrane for chain transfer (see signal hypothesis, Blobel and Dobberstein, 1975; amphiphatic tunnel hypothesis, Rapoport, 1985; translocator protein hypothesis, Singer et al., 1987*a*,*b*), then the signal sequence could be involved in its assembly or "opening" but apparently not for keeping it together or open until chain transfer is completed (as suggested in Singer et al., 1987*a*). Similarly, when considering models in which the chain transfer occurs directly through a lipid membrane (see the helical hairpin hypothesis, Engelman and Steitz, 1981; direct transfer model, von Heijne and Blomberg, 1979; phospholipid channel hypothesis, Nesmayanova, 1982) the interaction of the signal sequence with the lipid bilayer could be of importance only at the stage of translocation initiation but not at the actual chain transfer step.

The possibility that our results about p62 protein translocation would be unique to the viral system and different from the general translocation process in the ER we find most unlikely. Several results from this and earlier works suggest that the signal sequence of the p62 protein functions much in the same way as cleavable ones do. Firstly, studies with a temperature-sensitive mutant of SFV, ts3, have shown that the signal sequence of p62 requires a free NH_2-terminal end for function (Hashimoto et al., 1981). At the nonpermissive temperature the ts3 mutant is defective in cleavage between the C and the p62 protein region of the protein precursor because of a mutation that inactivates the autoproteolytic activity of C. This defect results in a translocation negative phenotype for the p62 protein. Secondly, the p62 signal sequence has been shown to be SRP dependent. If the mRNA for the structural proteins of SFV is translated in vitro in a wheat germ–derived system that is supplemented with salt-washed (and SRP-deprived) membranes then p62 translocation is observed only in the presence of exogenous SRP (Bonatti et al., 1984). If SRP is supplemented without membranes then p62 translation is arrested. Thirdly, our time course study about p62 synthesis and glycosylation in this work clearly demonstrates that the p62 chain is translocated cotranslationally across the ER membrane. This was also suggested by earlier studies in vitro (Garoff et al., 1978; Bonatti et al., 1984). In these studies it was shown that both microsomal membranes as well as SRP have to be added to the synthesis mixture before extensive lengths (~100 amino acid residues) of the p62 chains have been translated.

It is also possible to speculate on a mechanism in which the p62 signal sequence would be released from a putative translocation site by being replaced by another signal sequence-like structure in the p62 polypeptide. However, such a "rescue" mechanism appears improbable as the p62 signal sequence was found to be glycosylated early during translation of both the p62 polypeptide as well as the signal sequence-dhfr hybrid chain.

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Chapter 24

Formation and Function of the Semliki Forest Virus Membrane

Henrik Garoff, Peter Liljeström, Kalervo Metsikkö, Mario Lobigs, and Johanna Wahlberg

A large number of viruses have acquired an envelope or membrane for the purpose of transmitting their genomes between cells (Dubois-Dalcq et al., 1984; Simons and Fuller, in press). These viruses direct the synthesis of membrane proteins that are destined for either the plasma membrane or different compartments of the exocytic pathway in the infected cells. The membrane proteins have the capacity to recognize encapsidated genome structures in the cell cytoplasm and organize themselves together with the lipid membrane around these structures. This process, which is called virus budding, results in transfer of the viral nucleic acid from the cytoplasm of an infected cell in the form of the virus particle. The viral membrane proteins then direct the targeting of the virus particle to a new host cell and also guide the entry of the viral nucleic acid into the cytoplasm of the same. The latter event occurs through membrane fusion. In this process, the viral membrane proteins induce the fusion of the viral and the host membranes, releasing the encapsidated nucleic acid into the cytoplasmic space.

To study the mechanisms of viral membrane formation and fusion, we have used Semliki Forest virus (SFV), an enveloped virus belonging to the group of alphaviruses (Garoff et al., 1982). SFV has a single-stranded RNA genome of positive polarity. The genome directs the synthesis of a polymerase for replication of genomic (11.5-kilobase or 42S) and subgenomic (4.1-kilobase or 26S) RNA molecules (Fig. 1). The 26S RNA molecule is used for synthesis of the viral structural proteins. These are the capsid (C) protein and the membrane proteins p62 and E1, which are translated from a common coding unit of the 26S RNA in the order C-p62-E1 (see upper part of Fig. 2). The newly synthesized C molecule is used for nucleocapsid with genomic RNA in the cell cytoplasm, whereas the membrane proteins become inserted into the membrane of the endoplasmic reticulum (ER) for further transport to the plasma membrane. Virus budding occurs on the cell surface through association of the nucleocapsids with the viral membrane proteins. SFV entry into a new cell starts with the binding of the particle to some still uncharacterized structure on the cell surface. Next, the particle is internalized via the pathway of receptor-mediated endocytosis. Within the acidic milieu of the endosomes, the membrane proteins of the virus trigger the fusion reaction between the viral and the endosomal membranes (Kielian and Helenius, 1986; Marsh and Helenius, 1989). In recent studies, we have characterized (i) how SFV can direct the synthesis of both cytoplasmically destined C protein and several membrane proteins from a common coding unit, (ii) how membrane protein oligomerization regulates various steps in the assembly processs of the viral membrane, and (iii) how fusion is regulated by the state of the virus membrane-protein association.

NOVEL USE OF SECRETORY SIGNALS DURING BIOSYNTHESIS OF THE VIRAL MEMBRANE PROTEINS

The p62 and E1 proteins represent typical single-spanning membrane proteins which after

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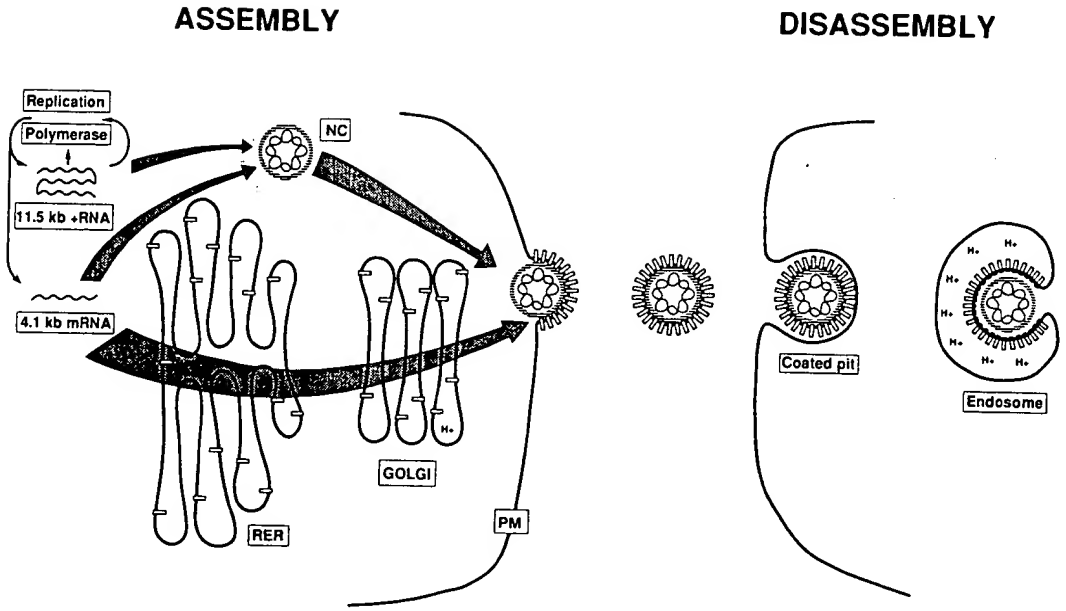

FIGURE 1. Schematic view over the main assembly and disassembly events involved in the life cycle of SFV. kb, Kilobase; NC, nucleocapsid; RER, rough endoplasmic reticulum; PM, plasma membrane.

synthesis have most of their molecular mass, including the N termini, within the ER lumen. In general, such membrane proteins are made with the aid of an N-terminally located cleavable signal peptide (or chain translocation signal) and a C-terminally located membrane anchor (von Heijne, 1985). However, the fact that p62 and E1 of SFV are translated after the C protein from a common coding region implies that internal chain translocation signals must be used in this system.

We have used gene technology and in vitro transcription-translation-translocation assays to localize and characterize the signal peptides of SFV. The results are summarized in Fig. 2. The signal peptide for the p62 protein is contained within the first 16 residues of this protein. Like cellular signal peptides, it requires a free N-terminal end to function, and this is achieved by the autoproteolytic removal of the preceding C chain. Interestingly, the p62 signal peptide is not removed by signal peptidase cleavage. Instead, this region is translocated into the lumen of ER together with the rest of the p62 luminal domain and becomes glycosylated at Asn-14.

The signal peptide for the E1 protein resides in the C-terminal part of the short transmembrane peptide called 6K, which is translated between the p62 and E1 proteins from the 26S RNA (Melancon and Garoff, 1986). Analogously, the insertion of the 6K peptide into the membrane is ensured by a signal peptide at the C-terminal region of the p62 protein. The E1 and the 6K signal peptides are clearly different from those of the p62 protein and other secretory proteins of the cell in the sense that they function in an internal location. However, it should be mentioned that both the 6K and E1 signal peptides are located very close after the anchor sequences of the p62 and 6K proteins, respectively. Thus, the membrane anchoring of the N-terminally located flanking sequences of these signal peptides could possibly prevent inhibition of signal peptide expression by preceding protein sequences. Moreover, since these flanking transmembrane peptides anchor the nascent chain to the ER membrane, it is possible that translocation of 6K and E1 is a signal recognition peptide-independent process.

The signal peptides of 6K and the E1 protein are each followed by typical cleavage sites for the signal peptidase (von Heijne, 1985). Indeed, by creating specific amino acid substitutions at the signal peptidase cleavage sites, we were able to abolish luminal cleavage of the polyprotein. The cleavages by the signal peptidase have two important consequences for SFV membrane protein biosynthesis. First, the p62-6K-E1 region is processed into individual protein chains. Second, the hydrophobic signal peptides are left at the C-terminal regions of the p62 and 6K chains, respectively. Thus, all signal

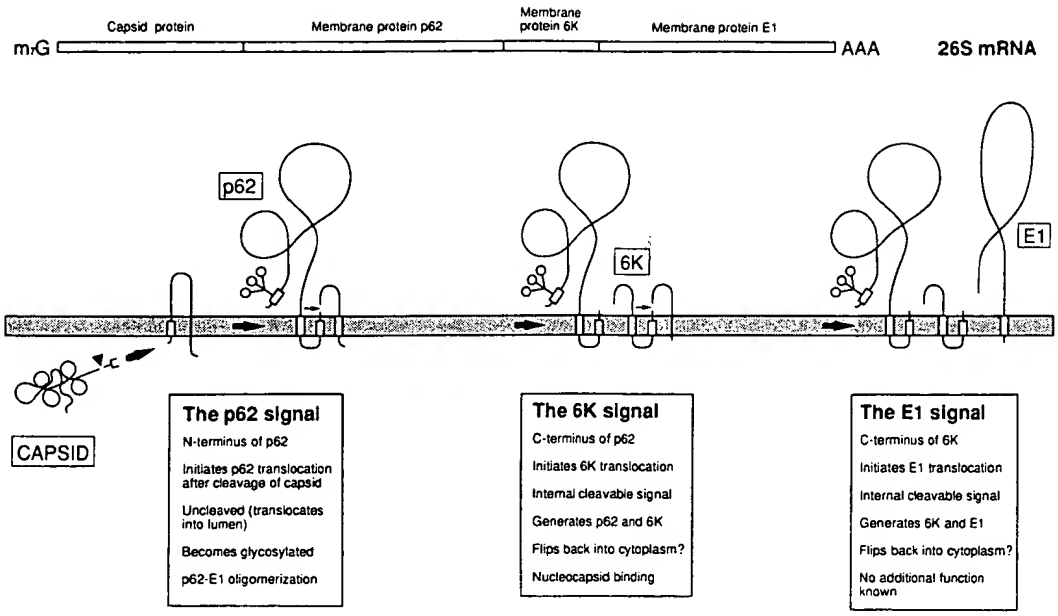

FIGURE 2. Use of translocation signals during synthesis of the structural proteins of SFV. Top, The gene map of the 26S subgenomic RNA. Middle, The process of membrane translocation of the p62, 6K, and E1 proteins. Small arrows on the luminal side denote signal peptidase cleavages. At the bottom, the characteristics of the three signal peptides are given.

peptides of the SFV membrane proteins are retained in the processed proteins. This probably reflects their need for further functions in virus assembly or entry.

OLIGOMERIZATION CONTROL OF MEMBRANE PROTEIN TRANSPORT

The p62 and E1 membrane proteins undergo efficient heterodimerization soon after translocation into the ER (Ziemiecki and Garoff, 1978; Ziemiecki et al., 1980; Rice and Strauss, 1982). The heterodimers are then transported to the cell surface to become incorporated into viral membranes. During a late stage of this transport, the heterodimer undergoes a proteolytic maturation: the p62 chain is cleaved after Arg-66 to yield the transmembrane E2 protein and the small E3 peptide. Viral particles contain essentially only the mature E2E1 heterodimers in their membranes.

The heterodimeric structures have been verified in several ways. These include (i) solubilization with nonionic detergents and subsequent immunoprecipitation with monoclonal antibodies against p62 (E2), E1, or both; (ii) solubilization as described above, followed by sedimentation analysis in sucrose gradients, and (iii) covalent intermolecular cross-linking and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

When the p62 and E1 proteins were expressed from separate coding units of engineered cDNA sequences, it became apparent that the heterodimerization exerts a profound control on the transport process of the membrane proteins; expression of only the E1 protein resulted in its complete retention in the ER compartment. In contrast, the p62 protein was transported to the cell surface even in the absence of E1. When E1 and p62 were coexpressed in the same cell but from different coding units, restoration of the E1 transport defect was observed. Thus, these experiments clearly show that E1 needs the p62 protein for cell surface transport. They also demonstrate most convincingly that p62E1 heterodimerization has to occur in the ER and in no other compartment of the exocytic pathway.

MEMBRANE PROTEIN OLIGOMERIZATION CONTROL OF THE MEMBRANE-NUCLEOCAPSID ASSOCIATION

During virus budding, interactions between the viral membrane proteins and the nucleocap-

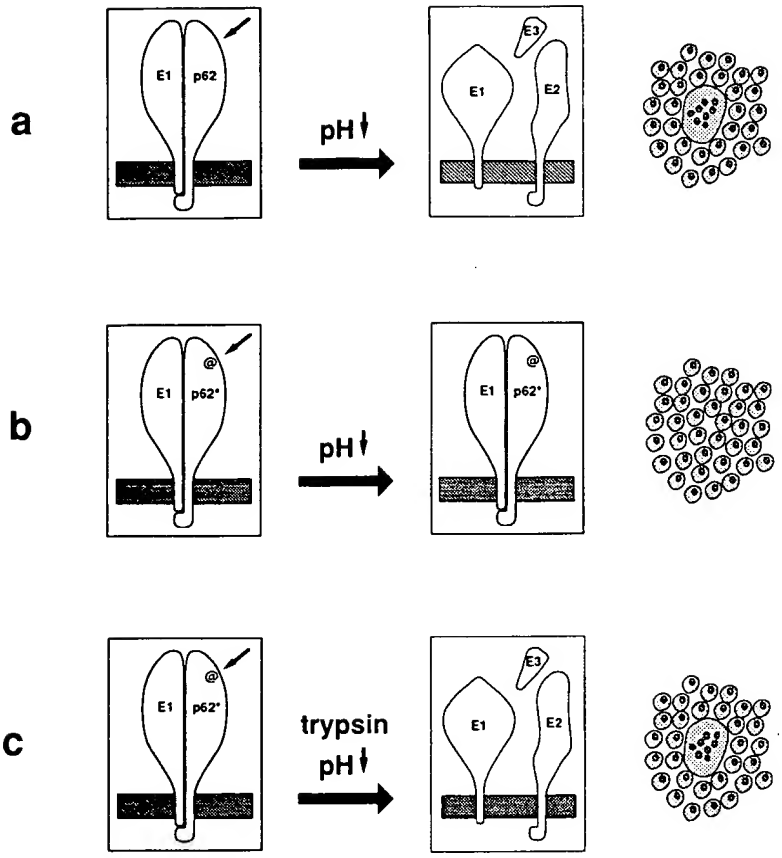

FIGURE 3. Schematic representation of cleavage activation of the fusion function of the SFV heterodimer. (a) Wild-type fusion phenotype. The envelope glycoprotein precursor p62 is proteolytically cleaved to E2 and E3 during transport to the cell surface. Low-pH treatment induces a fusion-competent conformation of the spike heterodimer, triggering cell membrane fusion and polykaryon formation. (b) Fusion phenotype of a p62 cleavage-deficient mutant. Low-pH treatment does not induce polykaryon formation. Fusion can, however, be restored by cleavage with exogenous trypsin of the mutant p62 (c), demonstrating that the p62 cleavage activation is essential for the acid-induced fusion function of the SFV spike heterodimer.

sid are required to curve the membrane around the whole particle. These interactions must primarily be based on interaction between the cytoplasmic or internal portions of the membrane proteins and the nucleocapsid surface. An examination of the cytoplasmic portion of the two membrane proteins of SFV shows that the p62 (E2) protein has 31 residues (including the 6K signal region) and that E1 has only 2 residues (both arginines) on this side of the membrane layer (Garoff et al., 1980). Therefore, it appears that only the p62 (E2) protein has enough molecular mass on the cytoplasmic side of the membrane for nucleocapsid interaction. An important corollary of this is that the E1 protein needs the p62 (E2) protein also for incorporation into virus particles.

Recently, the interaction between the p62 (E2) cytoplasmic peptide and the nucleocapsid has been verified in two ways. First, Vaux et al. (1988) showed, by using anti-idiotypic antibodies, that the two surfaces display complementarity. Second, we have demonstrated direct binding between a synthetic peptide corresponding to the p62 (E2) cytoplasmic domain and isolated nucleocapsids. Most interestingly, the peptide was able to bind only if presented in an oligomeric form or if bound to a solid matrix. This finding suggests that single p62 (E2) cytoplasmic domains are not sufficient for binding but that

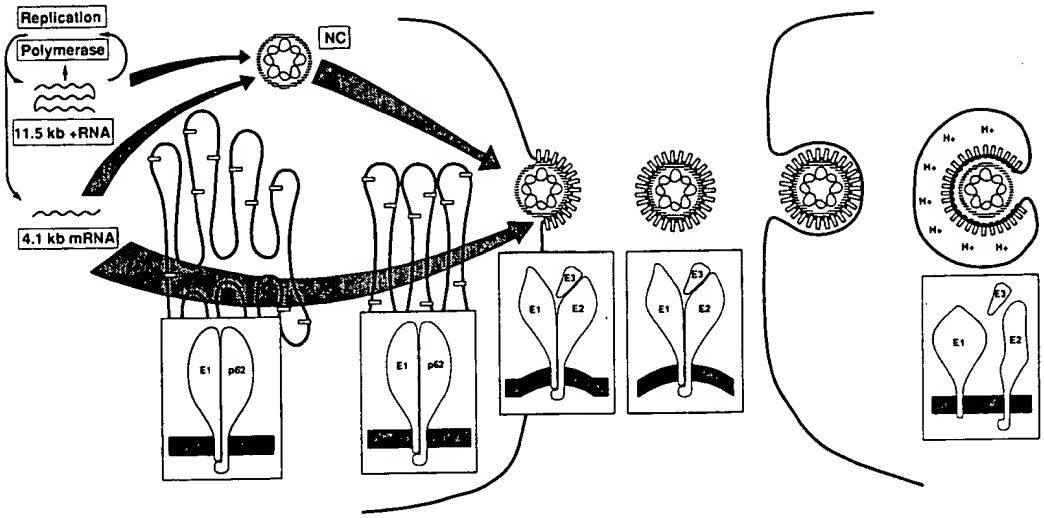

FIGURE 4. Regulation of SFV spike heterodimer association by p62 cleavage and pH. After translocation into the ER, the membrane proteins are transported through mildly acidic compartments to the plasma membrane as acid-resistant p62E1 heterodimers. After cleavage of p62, the virus particles, now containing E2E1 complexes, are released through budding. However, the association between E2 and E1 is sensitive to acidic pH. During entry of the virus through endocytosis, the acidic milieu of the endosome triggers the dissociation of the spike complex, resulting in fusion of the viral and endosomal membranes and release of the nucleocapsid (NC) into the cytoplasmic compartment. kb, Kilobase.

multivalent binding sites must be created. The suggestion finds further support in the recent cryoelectron microscopic analyses of both SFV and Sindbis virus (Vogel et al., 1986; Fuller, 1987). These studies have shown that the spike structures of the alphaviruses are formed from three copies of the E2E1 heterodimer. It could be that productive budding is driven only through the association of these $(E2E1)_3$ structures with the nucleocapsid.

OLIGOMERIZATION CONTROL OF ENTRY FUNCTIONS

Virus budding and fusion represent large opposite processes that normally follow each other during virus replication. An important question is how these processes are regulated so that they do not interfere with each other. One key factor in many other membrane viruses appears to be the synthesis of a fusion protein in an inactive precursor form (see, e.g., Boulay et al. [1987]). Proteolytic conversion of the precursor into a fusion-competent form usually takes place just before virus budding. Could the p62 precursor protein of SFV represent such a regulatable fusogen? This does not seem to be very likely because, as already discussed, this subunit appears to have a key function in membrane assembly. In contrast, there is good indirect evidence suggesting that the E1 protein carries the fusion activity. First, Omar and Koblet (1988) have created SFV particles essentially free of p62 by proteolysis and showed that these E1 viruses are both fusogenic and infectious. Second, Boggs et al. (1989) have characterized a Sindbis virus variant with a changed pH optimum for fusion and found that the phenotype can be correlated with amino acid substitutions in the E1 polypeptide. Third, Kielian and Helenius (1985) have shown that, in contrast to the E2 protein, the E1 protein gains protease resistance upon exposure to mildly acidic pH. Therefore, it could be that the p62 protein and its cleavage are totally unrelated to the fusion function of SFV.

To test this, we created cleavage-deficient mutants of the p62 protein by changing the Arg-66 residue at the cleavage site to either Leu or Glu. When these mutants were expressed from a 26S cDNA together with the other structural proteins in BHK cells, no cleavage of pulse-labeled p62 could be demonstrated even

after 3 h of chase. However, exogenously added trypsin was able to cleave the mutated p62 forms into apparently authentic E2. This cleavage probably occurred at one of the two remaining basic residues at the cleavage site. The trypsin assay enabled us to demonstrate that most of the mutant p62 protein was transported to the cell surface. Using our coimmunoprecipitation assay, we were also able to show that essentially all membrane protein subunits were present as p62E1 heterodimers.

To test the fusion activity of the p62 cleavage site mutants, we injected wild-type or mutant cDNA constructions into adjacent BHK cells on cover slips and then incubated the cells to allow surface expression of the membrane proteins. Fusion activity was assayed by screening for polykaryon formation after activation of potential fusogenic activity of viral membrane proteins by a 1-min incubation at pH 5.5 (Kondor-Koch et al., 1983). The results were clear-cut: neither of the mutants induced polykaryon formation (Fig. 3). Fusion could, however, be induced by the mutants if the mutants were treated with exogenous trypsin before the low-pH incubation to allow p62 cleavage. Hence, the results showed that p62 cleavage is essential to activate the fusion function of the SFV spike.

How then can the p62 (E2) protein affect the fusogenic activity if this is a property of the E1 protein? The most reasonable explanation is that p62 cleavage affects subunit association in the heterodimer. One simple possibility is that the mature heterodimer E2E1 has become sensitive to acid-induced dissociation and that the E1 protein must be free from p62 or E2 in order to function in fusion. Support for this model was obtained when we studied the strength of the heterodimeric association of the precursor and the mature form of the heterodimer in buffers of decreasing pH. The coimmunoprecipitation analyses demonstrated a marked resistance to dissociation of the p62E1 complex, whereas the mature E2E1 dissociated already at pH 6.4. Thus, the p62 (E2)E1 oligomerization reaction seems to be a major regulator both for the process of membrane assembly and for the fusion reaction during virus entry. These features are schematically summarized in Fig. 4.

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Internally Located Cleavable Signal Sequences Direct the Formation of Semliki Forest Virus Membrane Proteins from a Polyprotein Precursor

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The proteolytic processes involved in the cotranslational production of the Semliki Forest virus proteins p62, 6K, and E1 from a common precursor polypeptide were analyzed by an in vitro translation-translocation assay. By studying the behavior of wild-type and mutant variants of the polyprotein, we show that the signal sequences responsible for membrane translocation of the 6K and E1 proteins reside in the C-terminal regions of p62 and 6K, respectively. We present evidence suggesting that the polyprotein is processed on the luminal side by signal peptidase at consensus cleavage sites immediately following the signal sequences. Our results also lead us to conclude that the 6K protein is a transmembrane polypeptide with its N terminus on the luminal side of the membrane (type I). Thus, the production of all three membrane proteins is directed by alternating signal and stop-transfer (anchor) sequences that function in translocation and cleavage of the virus precursor polyprotein. This also shows conclusively that internally located signal sequences can be cleaved by signal peptidase.

Enveloped mammalian viruses have learned to utilize the biosynthetic transport pathway of their host cells as a means of synthesizing their own membrane components. Because of this feature, these viruses have become very useful model systems with which to study membrane formation in mammalian cells. For instance, the viral models have been involved in much of the pioneering work on membrane protein structure and synthesis at the endoplasmic reticulum (ER). Viral membrane proteins were among the very first examples of monotopic (influenza virus HA, vesicular stomatitis virus G) and polytopic (coronavirus E1) membrane proteins that were characterized in structural and topological detail (2, 24, 32, 45). Some of the monotopic membrane proteins were found to be inserted into the membrane of the ER in an N-terminus-in/C-terminus-out orientation through the sequential expression of an N-terminal signal sequence followed by a stop-transfer (or anchor) sequence (type I membrane proteins such as influenza virus HA and vesicular stomatitis virus G) (36, 38). Other monotopic membrane proteins were found to be inserted in the opposite orientation via combined signal-anchor sequences usually located at or close to the N-terminal end of the polypeptide chain (type II membrane proteins such as influenza virus NA) (7, 33).

We have been interested in the way in which the membrane proteins of Semliki Forest virus (SFV), an alphavirus, are synthesized. In contrast to most other membrane proteins, those of SFV are generated from a common coding unit on a 26S (4.1-kb) mRNA which includes all structural proteins of the virus. The cytoplasmic nucleocapsid (NC) protein C (33 kDa) is made first, followed by the membrane proteins p62 (62 kDa), 6K (6 kDa), and E1 (50 kDa) (for reviews, see references 18 and 40). This polyprotein system introduces several interesting questions about membrane protein topogenesis, such as (i) the nature of the signal sequences which are able to initiate or reinitiate polypeptide chain translocation at internal positions of the (nascent) polyprotein precursor and (ii) the kinds of cleavage events that are required to separate the individual proteins on the polyprotein sequence.

To this end, we and others have shown that the C protein is released from the nascent precursor chain by autoproteolysis (1, 22, 23, 35), thereby revealing an N-terminal signal sequence which is used for p62 chain translocation (6, 16, 19). Furthermore, we recently characterized a hydrophobic peptide in the C-terminal region of the 6K protein which can direct the translocation of the E1 protein as well as that of a heterologous protein (34). In the present work we have shown that this hydrophobic peptide is also required for E1 translocation in the context of the complete structural polyprotein and that the 6K-E1 cleavage site is sensitive to changes violating the consensus features of the signal peptidase cleavage site. Results are also presented which suggest that the 6K protein carries a functional stop-transfer signal and that the C-terminal region of the p62 protein (which precedes the 6K protein on the polyprotein sequence) functions as a signal sequence for the 6K protein. Therefore, we suggest that the 6K protein represents a type I transmembrane molecule like the two larger SFV membrane proteins and that in addition to the N-terminal signal of p62, two internal cleavable signal sequences are involved in the generation of the SFV membrane proteins.

MATERIALS AND METHODS

Bacteria, phage, and plasmids. *Escherichia coli* strains used were DH5α (Bethesda Research Laboratories), which is *recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lacZYA-argF*)*U169* ϕ80d*lacZ*Δ(M15); GM161 (*E. coli* CGSC 6476), which is *thr-1 leuB6 dam-4 thi-1 hsdS1 lacY1 tonA21 supE44*; DH5αF'IQ, which is *endA1 hsdR1 supE44 thi-1 recA1 gyrA96 relA1* ϕ80d*lac*Δ(M15) Δ(*lacZYA-argF*)*U169*/F' *proAB lacI*^{q} *lacZ*Δ(M15) TN*5* (Bethesda Research Laboratories); and RZ1032 (30), which is Hfr KL16 *dut-1 ung-1 thi-1 relA1 supE44 zbd-279*::Tn*10*. Bacteriophage M13mp19 (46) and plasmids pBR322 (5) and pGEM1 (Promega Corp.) have been described previously.

Materials. Most restriction enzymes, DNA polymerase I,

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Klenow fragment, calf intestinal phosphatase, T4 DNA ligase, and T4 polynucleotide kinase were from Boehringer, Mannheim, Federal Republic of Germany. *Sph*I, *Stu*I, and *Kpn*I, together with RNase inhibitor (RNasin) and SP6 polymerase, were from Promega Biotec, Madison, Wis. Sequenase (Modified T7 polymerase) was from United States Biochemical, Cleveland, Ohio. Proteinase K was from Merck, Darmstadt, Federal Republic of Germany. Ribonucleotides, deoxyribonucleotides, dideoxyribonucleotides, and the cap analog $m^7G(5')ppp(5')G$ were from Pharmacia. Oligonucleotides were produced by using an Applied Biosystems synthesizer 380B followed by high-pressure liquid chromatography (HPLC) and NAP-5 (Pharmacia) purification. Spermidine, phenylmethylsulfonyl fluoride, diethylpyrocarbonate, bovine serum albumin (BSA), creatine phosphate, and creatine phosphokinase were from Sigma, St. Louis, Mo. Pansorbin was from Calbiochem, La Jolla, Calif. Agarose was purchased from FMC Bioproducts, Rockland, Maine, and acrylamide was from Bio-Rad, Richmond, Calif. The glycosylation acceptor peptide *N*-benzoyl-Asn-Leu-Thr-*N*-methylamide and the nonacceptor peptide *N*-benzoyl-Asn-Leu-(*allo*)Thr-*N*-methylamide were synthesized as described previously (14). Reticulocyte lysate was prepared from rabbits as described previously (25). Canine pancreas rough microsomes (50 A_{280} units/ml) were prepared as described previously (26). ^{14}C-labeled proteins used as molecular weight markers were from DuPont, Dreieich, Federal Republic of Germany. L-[^{35}S]methionine and [α-^{35}S]dATP-α-S were from Amersham.

DNA methods. Plasmids were isolated essentially by using the alkali-sodium dodecyl sulfate (SDS) method (4). All basic DNA procedures were done essentially as described previously (39). DNA fragments isolated from agarose gels (3) were purified by benzoyl-naphthoyl-DEAE (BND)-cellulose chromatography (41). Plasmids were purified by sedimentation through 1 M NaCl followed by banding in CsCl (39). Competent cells were prepared as described previously (10). DNA sequencing was performed by following the United States Biochemicals protocol for using Sequenase.

Construction of mutants. pGEM1-SFV plasmid (also called pG-SFV-15/5) (34) carrying the complete SFV 26S cDNA region coding for all structural proteins was used in production of wild-type RNA for translation (Fig. 1). For the construction of most mutant variants, we transferred the *Cla*I fragment spanning the relevant DNA region into M13 for mutagenesis. Since both *Cla*I sites in the SFV cDNA are followed by a C residue, they become methylated and cannot be cleaved when the plasmid is isolated from a normal bacterial host; therefore, the plasmid was grown in the *dam*-negative host strain GM161 to overcome this methylation. The *Cla*I fragment spanning the p62-6K-E1 joint regions was isolated from an agarose gel and recloned into the *Cla*I site of pBR322 to obtain unique flanking restriction endonuclease sites to be used for cloning into M13 (data not shown). From this plasmid the *Eco*RI-*Hin*dIII fragment was isolated and recloned into *Eco*RI-*Hin*dIII-cut M13mp19 replicative form (RF). The RF was transformed into RZ1032, and phage was grown for 6 h in the presence of uridine to incorporate uracil residues into the DNA (30). The phage was precipitated with polyethylene glycol, and the single-stranded DNA was isolated by phenol extraction. Appropriate phosphorylated oligonucleotides were hybridized and mutagenesis was performed by primer extension with Sequenase as described previously (30, 43). Mutant, in vitro-synthesized heteroduplex RFs were transformed into DH5αF'IQ, and the resulting phages were analyzed for the presence of mutations by sequencing the complete insert. The RFs of the correct mutants was isolated and cut with *Eco*RI and *Hin*dIII, and the DNA fragments were gel purified and recloned into pGEM1-SFV as a *Cla*I fragment via intermediate *Eco*RI-*Hin*dIII cloning in pBR322 and growth in the *dam* host GM161. The presence of the correct mutation was once more confirmed by sequencing the concerned regions of the pGEM1-SFV variants. The oligonucleotide 5'-TGTCGAATGTTCGTATGCGTGCGCCCGCGG was used to delete the 6K region, the oligonucleotide 5'-CACACTAG CAAAGTGCGCCCG was used to change the -1 alanine residue of the 6K signal sequence to a phenylalanine, the oligonucleotide 5'-GTAAGCTCTGTTGGTTGCCCC changed the -3 alanine residue of the E1 signal sequence to a phenylalanine, and, finally, oligonucleotide 5'-GGCGGTTGCCCC GCGGCTCAGTAGCACTCTAAAAGAAAGGCT changed the leucine 5 and the leucine 10 residues of the E1 signal sequence to arginines.

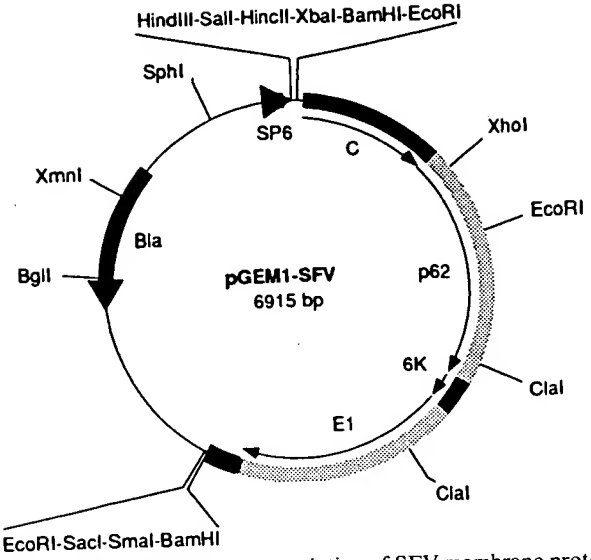

FIG. 1. Plasmid used for transcription of SFV membrane protein genes. pGEM1-SFV contains the complete subgenomic cDNA of the SFV, coding for all structural proteins (in the transcriptional order C-p62-6K-E1) of the virus. In vitro transcription starts at the promoter for the SP6 polymerase. The region flanked by the two *Cla*I sites was recloned for in vitro mutagenesis.

In vitro transcription and translation. Uncut CsCl-banded plasmid DNA was used as a template for in vitro transcription. RNA was synthesized at 37°C for 1 h in 10- to 50-μl reaction mixtures containing 40 mM Tris hydrochloride (pH 7.6), 6 mM $MgCl_2$, 2 mM spermidine hydrochloride, 5 mM dithiothreitol, 100 μg of nuclease-free BSA per ml, 1 mM each ATP, CTP, and UTP, 500 μM GTP, 1 U of RNasin per μl, and 100 to 500 U of SP6 RNA polymerase per ml. For production of capped transcripts, the analog $m^7G(5')ppp(5')G$ was included in the reaction at 1 mM (29). In vitro translation reactions with a rabbit reticulocyte lysate was performed at 30°C essentially as described previously (34): 1.5 μl of the in vitro-synthesized RNA was translated in a total volume of 15 μl with or without 2 μl of dog pancreas ER microsomal membranes. Potassium, magnesium, and spermidine concentrations were 100, 1.2, and 0.375 mM, respectively. Competing (acceptor) or control (nonacceptor) peptides for N-linked protein glycosylation reactions were added to a final concentration of 200 μM (16). For protease

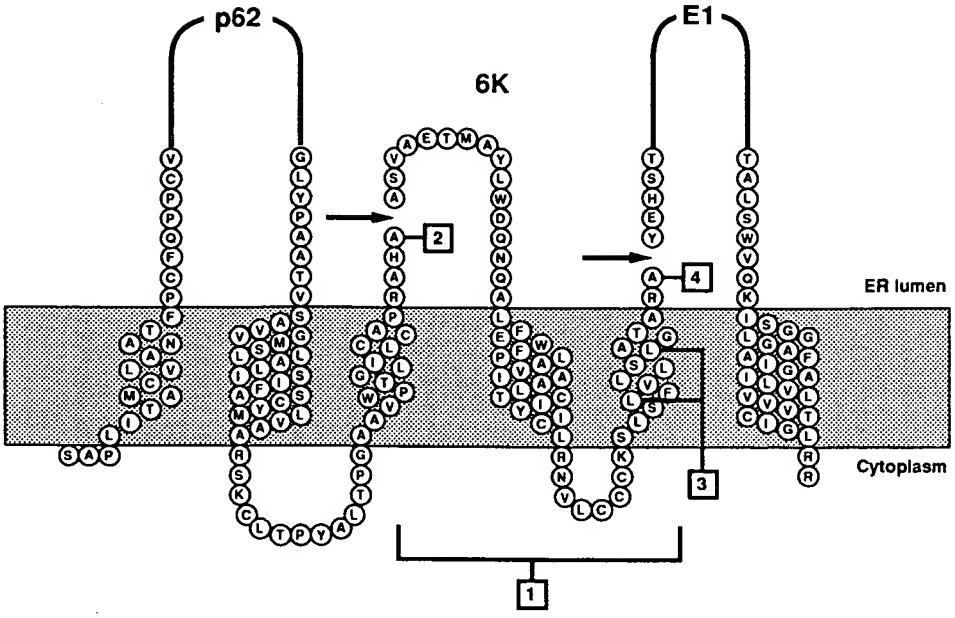

FIG. 2. Schematic presentation of the proposed cleavage events leading to the generation of the SFV structural proteins and their final topology in the ER membrane. The amino acid residues that were changed by site-directed mutagenesis in this study are marked by mutation numbers 1 to 4. Signal peptidase cleavages are indicated by arrows. Most of the luminal portions of the p62 and E1 proteins are drawn as solid lines and are not to scale.

protection experiments, proteinase K was added to a final concentration of 0.5 mg/ml and the samples were incubated at 0°C for 30 min in the presence or absence of 1% Triton X-100. Proteolysis was stopped by adding phenylmethylsulfonyl fluoride to 400 μg/ml, and samples were kept at 0°C for an additional 5 min before being processed for electrophoresis.

Gel electrophoresis. Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared and run on 10% separating gels with a 5% stacking gel as previously described (11). For resolving the 6K peptide, a 10 to 20% linear acrylamide gradient gel was used. The gels were fixed in 10% acetic acid–30% methanol for 1 h before being exposed to Kodak XAR-5 film. When the gel was prepared for fluorography (8), it was washed for 30 min in 30% methanol after fixation and then soaked in 1 M sodium salicylate–30% methanol for 30 min before being dried. Nucleic acids were run on agarose gels with 50 mM Tris-borate–2.5 mM disodium EDTA as the buffer. For staining, 0.2 μg of ethidium bromide per ml was included in the buffer and gel during the run. Sequencing gels were run as described previously (39) with Tris-borate as the buffer.

RESULTS

The C-terminal region of p62 contains a signal sequence. Since our model (35) predicted that the 6K protein is integrated into the ER membrane, we wanted to define the region of the polyprotein responsible for its translocation. The site of cleavage between p62 and 6K has been defined by amino acid sequence analysis (27), and this, together with the hydrophobic character of the tail of p62 (15), suggested that a signal sequence resides in the C-terminal part of p62. We reasoned that if this was the case and if we were to delete 6K by fusing the p62 and E1 coding regions in frame, translocation of E1 should still occur since the tail of p62 now would function as a signal sequence for E1. Therefore, using site-directed mutagenesis, we deleted the whole 6K gene from plasmid pGEM1-SFV (Fig. 2, mutation 1).

We first analyzed the behavior of the wild-type SFV polyprotein during in vitro translation. In vitro-synthesized RNA was translated in the absence of membranes, and two proteins with apparent sizes of about 100 and 30 kDa were produced (Fig. 3, lane 1). This corresponds well to the unglycosylated precursor protein consisting of p62, 6K, and E1 (107 kDa), while the smaller protein corresponds to the size of the capsid protein (C; 33 kDa). We conclude that faithful translation and cleavage of the nascent chain had occurred. Translation in the presence of microsomes produced protein species that corresponded closely to the expected sizes of glycosylated p62 (62 kDa), E1 (50 kDa), and 6K (6 kDa) (Fig. 3, lane 6; Fig. 4, lane 1). Addition of proteinase K showed that the p62 and E1 molecules had indeed been translocated, since their luminal parts were protected by the microsomal membrane (Fig. 3, lane 7). The protease treatment resulted in a small decrease in the size of p62, an indication of the trimming of its 31-amino-acid cytoplasmic tail. E1 did not change in size, since it has only two arginine residues exposed outside the microsomal vesicle, and therefore possible trimming could not be visualized by this assay. The untranslocated capsid protein was completely sensitive to added protease. The same was true for the remaining precursor form, since it was totally sensitive to protease, indicating that it was not translocated. The resistance of the membrane proteins to protease was due to inaccessibility, since protease treatment in the presence of Triton X-100 completely degraded all protein species (lane 8). During in vitro translation, p62 is glycosylated at four Asn positions while E1 is glycosylated only once (18). We used this fact to verify translocation by adding an excess of a competing acceptor peptide for N-linked glycosylation to the translation-translocation reaction mixture. Since distinct smaller forms corresponding to the sizes of unglycosylated

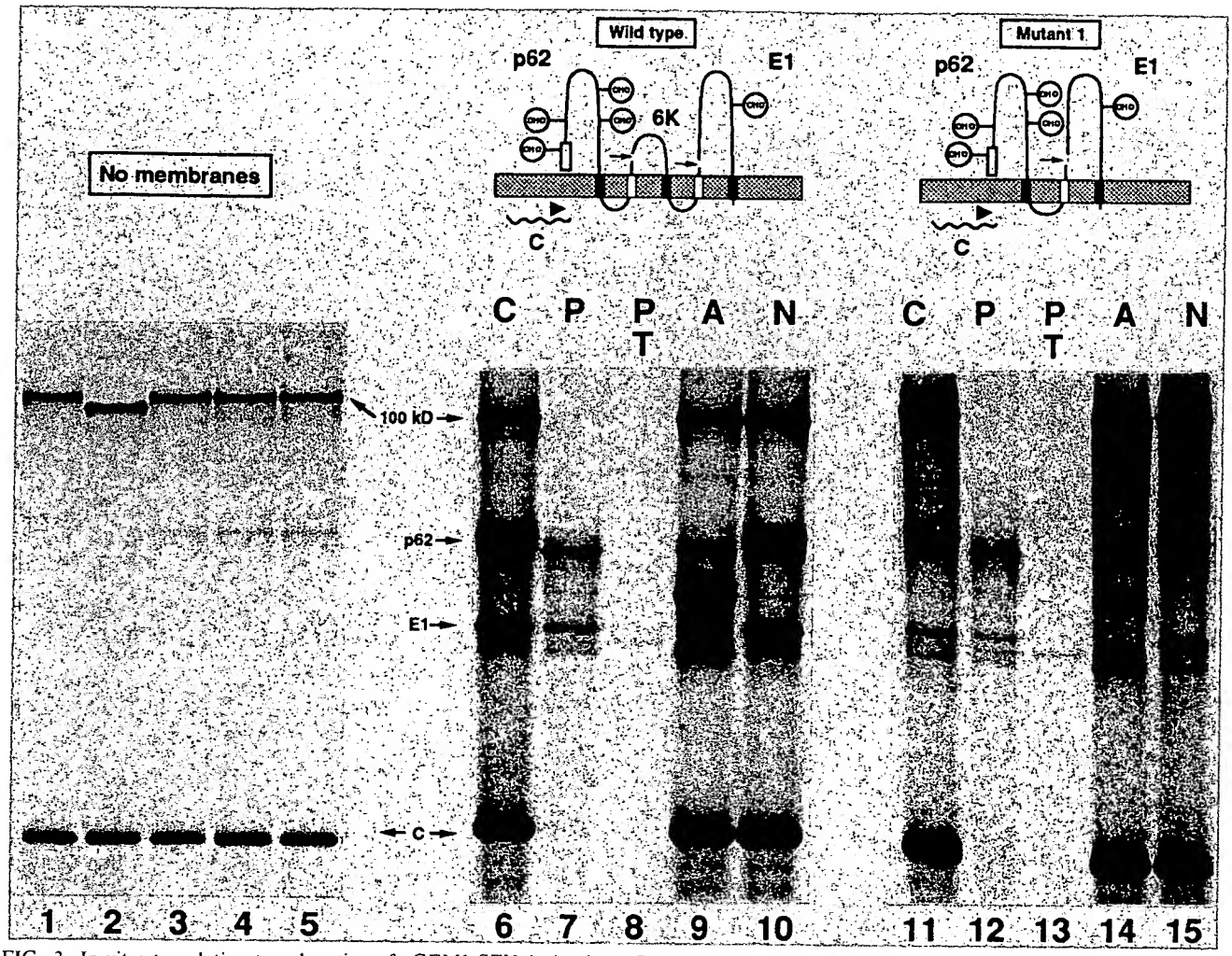

FIG. 3. In vitro translation-translocation of pGEM1-SFV derivatives. SFV 26S cDNA was transcribed in vitro (SP6), and the RNA was translated (rabbit reticulocyte lysate) in the absence or presence of microsomal membranes. The samples were analyzed by SDS-PAGE (10% acrylamide) and autoradiography. Treatment with protease (P) or protease in the presence of Triton X-100 (P/T) is indicated. Translations were also performed in the presence of an acceptor (A) or nonacceptor (N) peptide. Bands corresponding to the 100-kDa precursor, p62, E1, and C proteins are marked. Note that of the two faint bands below E1, the smaller represents unglycosylated E1 (compare, e.g, lanes 6 and 9) and the larger represents a tRNA-dependent (but ribosome-independent) addition of methionine to a rabbit reticulocyte lysate protein (25). This is resistant to protease digestion (see, e.g., lane 8). This makes the band an excellent internal marker, especially when judging the shifts in molecular weight of E1. Above each set of translation-translocation assays, the interpreted end result is depicted. Luminal arrows indicate signal peptidase cleavage. Glycosylation of p62 and E1 is marked by CHO. Symbols: ▶, cytoplasmically occurring capsid protease cleavage; ■, membrane anchors; □, signal sequences; ▨, E1 signal variant containing two arginine residues.

chains were found, we conclude that p62 and E1 were glycosylated and hence also translocated (lane 9). This inhibition was specific, since addition of a control peptide had no effect (lane 10).

In vitro-synthesized RNA from the 6K deletion construct was also translated in the absence of membranes. As expected, this construct gave a slightly smaller precursor protein, whereas production of the capsid protein was unaffected (Fig. 3, lane 2). In the presence of membranes, p62, E1, and C proteins of wild-type sizes were produced (Fig. 3, lane 11), whereas the 6K peptide was absent (Fig. 4, lane 2). In this case the p62 and E1 proteins were translocated and cleaved somewhat less efficiently than the wild-type construct. As in the case of the wild-type construct, translocation of the p62 and E1 species could be judged from their resistance to added protease (lanes 12 and 13) as well as their glycosylation (lanes 14 and 15). Some of the precursor form appeared to be translocated, since a minor portion of it had a slightly lower mobility than the rest of the precursor (lane 11), indicating that it had become glycosylated. By contrast, translation in the presence of an acceptor peptide resulted in a decrease in the apparent size of this precursor form (lane 14). Since neither of the precursors was totally protected from added protease, we conclude that the translocated (but uncleaved) portions became trimmed within the cytoplasmic loop domain of the p62 tail, resulting in protein species of p62 and E1 sizes comigrating with the appropriately cleaved (by signal peptidase) ones (lane 12).

Signal peptidase cleaves between p62 and 6K. Since it was evident from these results that the p62 tail does contain a functional signal sequence, it was of interest to define the mode of cleavage between p62 and 6K in the processing of

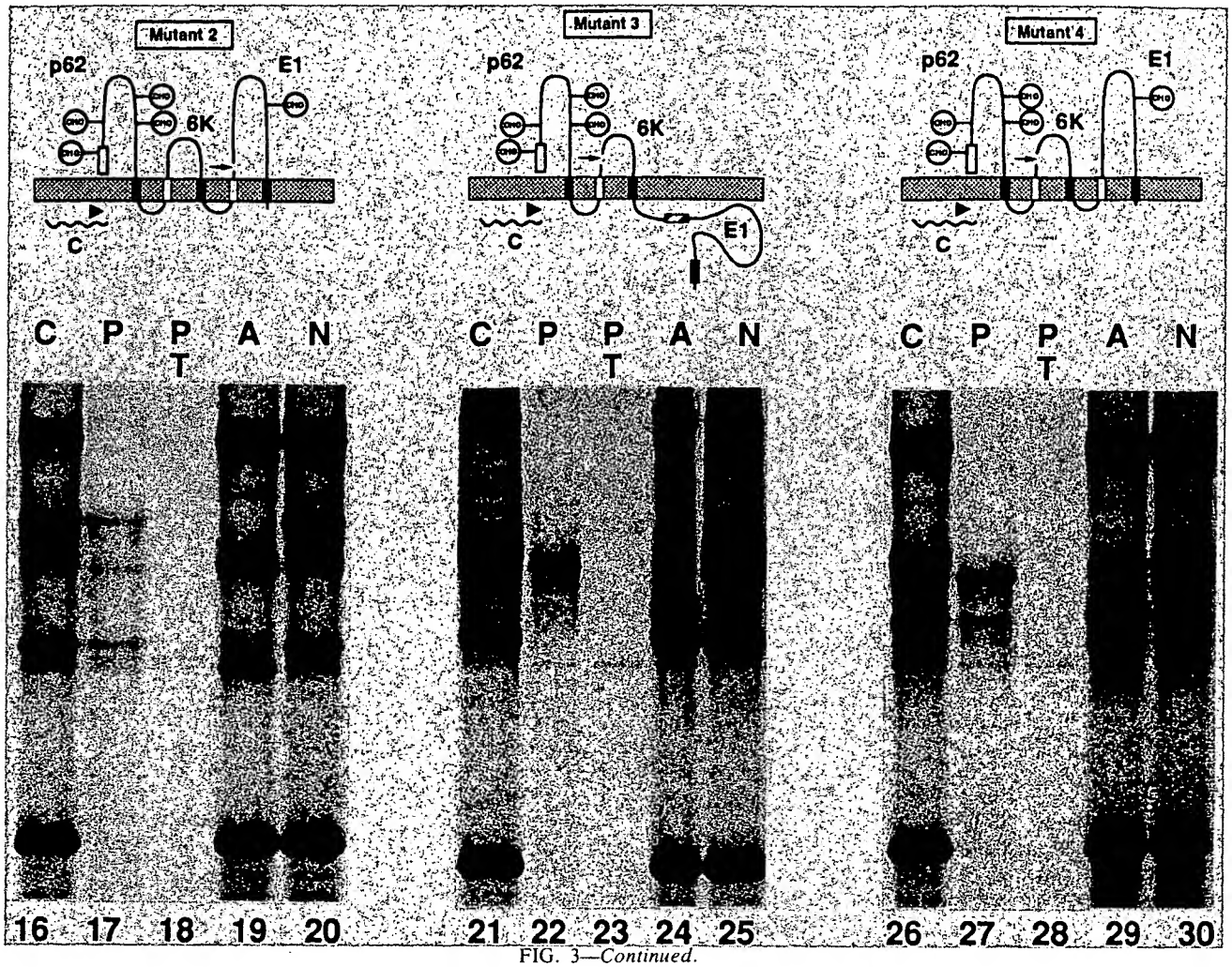

FIG. 3—*Continued.*

the SFV precursor protein. To this end, the ultimate C-terminal alanine residue of p62 was changed to a phenylalanine (Fig. 2, mutation 2). If cleavage is indeed performed by signal peptidase, this mutation should prohibit cleavage since the Phe residue is at position −1 of the putative signal peptidase cleavage site, a position that contains only small amino acid species (44). When translated in the absence of membranes, this mutant construct encoded 100- and 30-kDa protein species corresponding to p62-6K-E1 and C, respectively, suggesting that the substitution had no effect on translation per se (Fig. 3, lane 3). However, in the presence of membranes a p62 protein which was somewhat larger than the wild-type protein appeared, indicating that cleavage between p62 and 6K had not occurred (lane 16). Moreover, production of the 6K protein species could not be detected (Fig. 4, lane 3). Production of E1 was not affected by the mutation. Small portions of both the fusion protein and E1 remained unglycosylated (Fig. 3, lane 16), although they evidently had been translocated, since they were resistant to protease (lane 17). Protease treatment did not cleave the fusion protein, suggesting that since the p62 tail now was linked to the luminal part of 6K, it was perhaps pulled close to the microsomal membrane and hence not accessible to protease digestion. Translocation also resulted in glycosylation of the proteins, as verified by the addition of an acceptor peptide which inhibited glycosylation, resulting in smaller products (lanes 19 and 20).

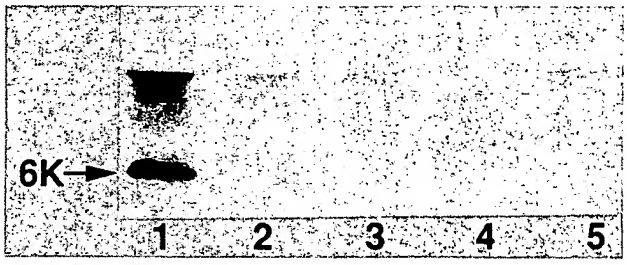

FIG. 4. In vitro translation-translocation analyzed on an SDS–10 to 20% gradient gel. Only the region below the globin area is shown. The position of the 6K protein is indicated. The wild-type construct (lane 1) and mutant variants 1 to 4 (lanes 2 to 5, respectively) are shown.

The 6K tail functions internally in the polyprotein as a signal sequence for E1. Having defined the mode of cleavage at the N-terminal border of 6K, we next wanted to characterize the signal sequence of E1. It was shown previously that the C-terminal region of the 6K protein can function as a signal recognition particle (SRP)-dependent signal sequence for the

translocation of E1 from a single coding unit or an unrelated reporter protein (34). However, it still remained unsettled whether this region also retains this function in its context as an internal signal within the polyprotein. To test this, we now used the complete coding sequence for the SFV proteins and changed the consensus features of the putative E1 signal sequence. We reasoned that if the hydrophobic character of the putative E1 signal sequence were changed to a more charged one, it would not function in translocation, thus creating a 6K-E1 fusion protein with an untranslocated and unglycosylated E1 part. Accordingly, two leucine residues (residues 5 and 10) within the E1 signal sequence were both changed to arginine residues (Fig. 2, mutation 3) and translocation assays were performed. The results were consistent with our predictions; i.e., in addition to a wild-type p62, an untranslocated and unglycosylated protein species was produced with a size corresponding to that of an unglycosylated 6K-E1 hybrid with about the same size as glycosylated E1 (Fig. 3, lanes 21 to 25). Finally, since we could not detect any 6K protein (Fig. 4, lane 4), we concluded that a fusion protein consisting of 6K and E1 had been produced.

The double arginine mutant (from mutation 3 in Fig. 2) was also used to address another important question, that of stop-transfer proficiency exerted by 6K. The fact that the mutation in the E1 signal completely abolished downstream translocation suggested that 6K contains a stop-transfer signal and that E1 translocation is reinitiated after 6K stop-transfer. If this were not the case, one would expect that the whole downstream stretch of the polyprotein would become translocated into the lumen of the microsomes and thus would be resistant to externally added protease, which it was not.

Since the C-terminal region of 6K contained signal sequence activity, we tested whether cleavage between 6K and E1 is performed by signal peptidase. Accordingly, we changed the putative cleavage site at the end of the 6K tail by changing the alanine residue at position −3 to a phenylalanine (Fig. 2, mutation 4). Translation in the absence of membranes showed that the mutant construct was faithfully translated (Fig. 3, lane 5). Membrane translocation assays showed that signal peptidase cleavage between 6K and E1 was indeed abolished. Translation in the presence of membranes produced a wild-type-size p62 while the E1 protein band increased in size, suggesting that 6K now was fused to E1 (Fig. 3, lane 26). At the same time, we could not detect any 6K protein (Fig. 4, lane 5). Again, translocation was verified by testing for protease resistance and occurrence of glycosylation (Fig. 3, lanes 27 to 30). The p62 tail was trimmed in a normal fashion, whereas the E1-6K fusion protein remained totally resistant. This result was somewhat surprising, since the external (putative) loop region between 6K and E1 should consist of 8 amino acids (Fig. 2) and we expected this loop to be cleaved. Apparently, the close vicinity to the membrane blocked digestion.

DISCUSSION

By using an in vitro transcription-translation-translocation system, we analyzed the cotranslational processing of the SFV structural polyprotein. More specifically, by studying the phenotypic expression of a panel of site-specific mutations, we defined the mode of events that lead to the cleavage and formation of the p62, 6K, and E1 proteins.

The C-terminal region of the 6K protein was previously shown to contain a signal sequence that is able to initiate translocation of E1 when expressed from a single coding unit (34). However, it remained unclear whether this region could function in a similar fashion when placed internally within the SFV structural polyprotein sequence. We found that translocation of downstream regions of the polyprotein was completely abolished when the hydrophobic character of the signal was removed. Luminal cleavage by signal peptidase was also inhibited when the cleavage site consensus structure was interrupted, strongly suggesting that the tail of 6K functions as a normal cleavable signal sequence for E1 and that it functions internally in the processing of the SFV polyprotein.

We believe that the 6K protein does indeed represent a transmembrane molecule, as proposed earlier (35). This is substantiated by several of our findings. First, we found that the C terminus of p62 contains a functional signal sequence. In the absence of 6K, this region was able to translocate the E1 protein through the membrane, showing that the region contains signal sequence activity. This notion is supported by the structural features of this area, which contains a stretch of uncharged and mostly hydrophobic residues that have the ability to form an α-helix (20, 44). Moreover, this region is highly conserved within the alphaviruses, in which almost all substitutions are conservative (9, 13, 15, 21, 28, 31, 42). Second, the three terminal amino acid residues preceding 6K form a consensus cleavage site for signal peptidase. Luminal cleavage between p62 and 6K was abolished when these consensus features were changed, strongly suggesting that cleavage is indeed performed by a signal peptidase and supporting the idea that the p62 tail functions as a signal sequence. The apparent cleavage of the nascent chain between p62 and 6K by signal peptidase is also good evidence for the initial translocation of the 6K polypeptide. Third, as described above, when the signal sequence of E1 was interrupted by two arginine substitutions, neither translocation nor glycosylation of the downstream polypeptide occurred, suggesting that a stop-transfer event occurred during insertion of the 6K protein. This means that the E1 signal would have to reinitiate E1 translocation. Further evidence stems from the fact that the 6K-E1 fusion protein generated by mutation 4 (cleavage site) was totally resistant to protease digestion; therefore, the major part of 6K must have been translocated through the membrane. All in all, we think that the apolar region between the glutamate (6K residue 21) and the next arginine (6K residue 37), together with the four hydrophobic residues (LFWL) preceding the glutamate, constitutes the anchor region of 6K (44).

The results from this work and earlier studies now allow us to propose a complete scenario by which SFV utilizes both viral and host proteases in combination with anchor domains and signal sequences to generate all its structural proteins. Translation of the 26S subgenomic RNA starts with the capsid protein in the cytoplasm of the host cell. Once the complete capsid protein has been made and folded, its intrinsic serine protease activity cleaves the nascent chain between a tryptophan and a serine residue to free the capsid protein from the translation complex (1, 22, 23, 35).

Cleavage of the nascent chain reveals the N-terminal signal sequence of the p62 moiety, which targets the translation complex (by virtue of its interaction with the SRP) to the ER membrane, initiating the cotranslational translocation of the polypeptide through the membrane (6, 19). The p62 signal sequence is unusual in the sense that it is not cleaved off by signal peptidase. Instead, it is released into the lumen of the ER, where it becomes glycosylated long

before synthesis of p62 has been completed (16). This shows that the signal sequence is required only for chain translocation initiation, but not for completion.

Translocation of the p62 protein is arrested when its anchor sequence inserts into the membrane (11, 12, 15, 17). The C-terminal region of p62, which is left on the external (cytoplasmic) side, contains a second signal sequence which reinitiates translocation of the 6K moiety of the growing polypeptide (see above). This process could be SRP independent, since the ribosome complex is already at the membrane and should not need membrane targeting. This notion is supported by the fact that a deletion mutant of the SFV polyprotein, lacking all downstream residues from residue 25 of 6K, can efficiently translocate and can be processed correctly between p62 and 6K (11). Since the ribosome covers some 40 residues of a growing polypeptide chain (a stretch which in this case spans the 6K signal as well as the first 24 residues of 6K), the translation complex would dissociate (and thus prohibit the SRP from binding) before the signal sequence of 6K emerges from the ribosome interior to react with the SRP, since at least 50 residues are needed before SRP-mediated translational arrest occurs (37).

The signal sequence of 6K is cleaved by signal peptidase (see above), and translocation continues until arrested by the insertion of the 6K anchor sequence into the membrane. Soon after this, translocation of E1 is reinitiated by the E1 signal sequence resident in the 6K tail (34) (see above). During translocation of E1, the signal sequence is cleaved off by signal peptidase (see above), and finally translocation is arrested when the E1 anchor reaches the membrane. The end result will be the generation of three transmembrane proteins, p62, 6K, and E1, with the topology shown in Fig. 2.

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Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line

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ABSTRACT

We report the development of an advanced system for transfer and expression of exogenous genes in mammalian cells based on Moloney murine leukemia virus (Mo MuLV). Extensive deletion/mutagenesis analysis to identify cis-acting signals involved in virus transmission has led to the design of a family of novel, highly efficient retroviral vectors and a partner helper-free packaging cell line. The pBabe retroviral vector constructs transmit inserted genes at high titres and express them from the Mo MuLV Long Terminal Repeat (LTR). Each of these vectors has been constructed with one of four different dominantly acting selectable markers, allowing the growth of infected mammalian cells in the presence of G418, hygromycin B, bleomycin/phleomycin or puromycin, respectively. The high titre ecotropic helper free packaging cell line, ΩE, was designed in conjunction with the pBabe vectors to reduce the risk of generation of wild type Mo MuLV via homologous recombination events. The ΩE cell line was generated with separate *gagpol* and ecotropic *env* expression constructs with minimal sequence overlap and decreased sequence homology achieved by 'codon wobbling'. Homologous *env* coding sequences were deleted from the pBabe vectors without diminishing recombinant vector titre. Together, the pBabe vectors and ΩE cell line should prove useful in experiments where highest frequencies of gene transfer, or concomitant expression of several different genes within a single cell are required with minimal risk of helper virus contamination.

INTRODUCTION

Helper free retroviral vector systems have been increasingly utilised in gene transfer experiments because they are designed for can cotransfer and expression of a gene plus a selectable marker (1, 2). Mo MuLV based retroviral vectors have been successfully applied to a number of studies including mutagenesis (3), promoter traps (4, 5), cell lineage analysis and oncogenesis within developing organs (6, 7) and are currently being used in the optimization of gene therapy regimes (8).

Existing retroviral systems however, still suffer from a number of limitations. First, some of the most widely employed retroviral vectors and packaging cell lines fail to produce recombinant titres as high as those of wild type Mo MuLV which are necessary for gene transfer into rare cell populations such as hematopoetic stem cells (9). Second, only a few retroviral vectors reproducibly express an inserted cloned gene after selection of the co-introduced selectable marker, and for the most part the selectable marker is limited to the *neo* gene from the Tn5 transposon which confers resistance to the aminoglycoside G418 in mammalian cells (10). Last, some vectors and packaging cell lines are more likely to interact in the generation of contaminating wild type Mo MuLV than others (11). Besides posing the hazard of viraemia in the host during gene therapy or whole animal experiments, such wild type virus leads to the mobilisation and spread of defective vector proviruses which can blur the precise interpretation of experiments usually afforded by the clonal nature of vector proviral integrations.

The goal of the work presented in this report was to produce a generally applicable Mo MuLV based retroviral vector system that would transmit and express inserted genes as efficiently as endogenous genes of Mo MuLV while minimising the risk of helper virus contamination.

MATERIALS AND METHODS

Construction of Retroviral Vectors

Subcloning was carried out via standard recombinant DNA techniques (12). Oligonucleotides were synthesised on an Applied Biosystems 380B and purified by denaturing acrylamide electrophoresis. Site directed mutagenesis was performed as described (13). Exact restriction maps and details of each of the constructs are available upon request.

Transfection, Retroviral Infection and Titration

Constructs were transferred into cells via calcium phospate mediated transfection employing a modified protocol (14) in

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which packaging cells were split onto 10 cm dishes at a density of 7.5×10^5 18 hour prior to transfection. 10 μg of construct DNA as a precipitate was applied to cells for 6 hours followed by 15% glycerol shock for 3 minutes with replacement of 1/2 normal volume of medium. Transiently produced virus was harvested 48 hours post glycerol shock.

Stable ecotropic packaging cell lines were generated by transfecting ecotropic packaging cell lines such as Ψ-2 (15) and ΩE with vector DNA, or infecting them with virus released from the transiently transfected amphotropic packaging cell line PA317 (16). Virus was obtained from stable producer cell lines by allowing the cells to reach confluence, removing their spent medium, replacing it with 1/2 volume of fresh medium and harvesting the conditioned medium 48 to 72 hours later.

Retroviral infections/titrations were performed by filtering producer cell culture supernatants through a 0.2 or 0.45 μm filter and adding polybrene to a final concentration of 8 μg/ml. Supernatants were added either neat or diluted into medium supplemented with 8 μg/ml polybrene before application in 2 ml volumes to a 10 cm dish that had been seeded with 7.5×10^5 cells 18−24 hour prior to infection. Infections proceeded for 2 hours, after which time the viral supernatant was removed and replaced with fresh medium. 24 hours later cells were split 1:20 into selective medium in 10 cm dishes, with medium changed every third day until drug resistant colonies were visible without the aid of microscopy. Drug selection levels used for vector infection of NIH 3T3, PA317, Ψ-2 and ΩE cells were as follows: G418: 1 mg/ml, hygromycin B: 50 or 150 (Ψ-2) μg/ml, phleomycin: 10−25 μg/ml, puromycin: 1.0−2.5 μg/ml.

Stable Expression Analysis: Northern Analysis, CAT Activity and v-Ha-*ras* Production

Total cellular RNA was prepared and subjected to Northern analysis according to standard protocols (12, 17). For quantitation of CAT activity, cells were grown just to the point of confluence on a 10 cm dish, then lysed in 300 μl of NP-40 buffer (0.65% v/v NP-40, 10 mM Tris-Cl pH 8.0, 1mM EDTA, 150 mM NaCl). Extracts were harvested and incubated at 68°C for 10 minutes before being assayed as described (18) and the results normalised for extract protein content (Protein Assay, BioRad).

Cells infected with a vector containing a v-Ha-*ras* gene were grown to 3/4 confluence, washed, starved in serum and methionine free medium for 30 minutes then metabolically labeled overnight with ^{35}S methionine in methionine free medium supplemented with 1% v/v FCS. Cells were lysed in phospholysis buffer (20 mM $NaPO_4$ buffer pH 7.5, 100 mM NaCl, 1% v/v Triton X-100, 0.5% w/v Na deoxycholate, and 0.1% w/v SDS) on ice for five minutes with periodic agitation. Cell lysates were sequentally incubated with rat monoclonal α v-Ha-*ras* antibody (Oncogene Science Inc.), rabbit α rat IgG (Cappel Labs), and protein A-Sepharose (Sigma). Bound material was denatured and electrophoresed through a 12.5% SDS polyacrylamide gel. The gel was fixed in10% v/v glacial acetic acid/40% v/v MeOH, incubated with 1M salycilic acid, washed in dH_2O, dried, and autoradiographed at −70°C with intensifying screen.

RESULTS

Effect of Splice Donor Mutations on Retroviral Vector Titre

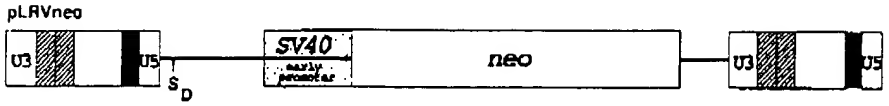
In the context of attempting to generate a retroviral vector that would most reliably express an inserted gene and a selectable marker it was decided to employ a DO (*D*irect *O*rientation) retroviral vector architecture (19). Here the promoter residing in the 5' LTR is used to transcribe the inserted gene and an internal promoter is used for expression of a drug resistance marker (although this gene arrangement can be reversed), with both transcripts sharing a common termination/polyadenylation signal in the 3' LTR. In such vectors it is advantageous to eliminate the unused splice donor in order to avoid artifacts, such as the activation of cryptic splice acceptors within inserted cDNAs (T. Claudio, personal communication).

Our initial attempts at assembling an efficient splice deficient DO vector (pLRV) led to the observation that sequence alterations in the splice donor region had adverse effects on the virus titre (Figure 1). pLRV was based on the unpublished pLJ construct whose design is essentially identical to the splice competent vector pDO-L (19) with the exception of an altered splice donor region (Figure 1) designed to inactivate the Mo MuLV splice donor. Unexpectedly, the recombinant titres of polyclonal Ψ-2 producer lines of both pLJ and pLRV were ten-fold lower (10^4 cfu/ml) than those of previous vectors such as pZipNeo SV(X) and pDO-L (10^5 cfu/ml). Since the only known variable introduced into the pLJ vector relative to pDO-L was the altered splice donor region, the possibility that this region might be responsible for the low titres associated with pLJ and its derivative pLRV was explored. The wild type Mo MuLV splice donor region was substituted for the mutant splice donor region of pLRV to generate pLRV S+, and the titres associated with polyclonal stable Ψ-2 producers of these vectors were quantitated. As the averaged results of two independent experiments in Figure 1 demonstrate, substitution of the wild type Mo MuLV splice donor region led to a six-fold increase in the titre of pLRV S+ over pLRV.

In order to generate a high titre DO type vector it was necessary to inactivate the Mo MuLV splice donor without adversely affecting vector titre. To this end, two point mutants of the Mo MuLV splice donor were made by site directed mutagenesis in M13 phage. The mutation designated (AGGT to AGAT) was designed to eliminate splicing by removal of the G residue which normally participates in lariat formation during splicing (20), while the 194 mutation (AGGT to AGGC) was chosen since it is present in the non-splicing acutely transforming defective retrovirus Harvey MuSV (21). Both mutations were introduced into the pLRV vector yielding pLRV 193 and pLRV 194. Titres of supernatants of polyclonal stable Ψ-2 producer cell lines of the vectors exceeded those of the producers of pLRV but not those of pLRV S+. Since the titres associated with pLRV 194 were the highest of the two vectors with point mutant splice donors, the 194 splice donor mutant was chosen for use in future DO retroviral constructs.

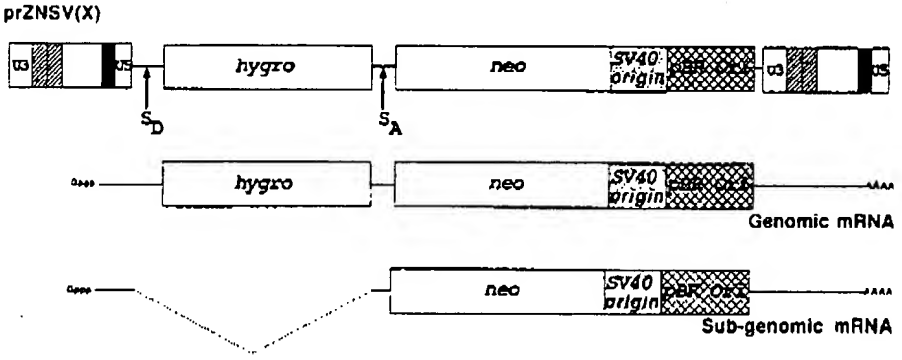
Functional Analysis of the 194 Splice Donor Point Mutation

To determine the degree to which the high titre 194 point mutation was effective in inhibiting splicing, it was introduced into the leader region of the splicing vector prZNSV(X) (a version of pZipNeoSV(X) with a simplified bacterial vector backbone with a limited number of restriction sites) to give prZNSV(X) 194. The *hph* gene which confers resistance to hygromycin B (22) was inserted into both prZNSV(X) and prZNSV(X) 194, and stable Ψ-2 producer cell lines established by selection in hygromycin B. Supernatants from both producer populations were used in titrations on NIH 3T3 cells using either G418 or hygromycin B selection. As shown in Figure 2, the G418^r titre associated with the spliced mRNA was diminished by three orders of magnitude in prZNSV(X) 194 compared to prZNSV(X), while



	splice donor sequence	single provirus polyclonal G418 titre cfu/ml
wild type	agGTaagctggc	3.4×10^5
194	agGCaagctggc	2.3×10^5
193	agATaagctggc	1.4×10^5
pLJ	agATaag····c	5.8×10^4

Figure 1. Effects of splice donor sequences on retroviral vector titre. The wild type Mo MuLV splice donor and two variants (created by site directed mutagenesis) were cloned into the vector pLRV. Each construct was transfected into PA317 cells and the transiently produced virus used to infect Ψ-2 cells, which were G418 selected to establish stable ecotropic producer lines. The supernatants of the four stable producer populations were used to infect NIH 3T3 cells. Subsequently the G418^r titres were quantitated. The results shown represent the average of two independent experiments.



Splice Donor	Hygro Titre	G418 Titre
Wild type	3.6×10^5	1.5×10^5
194	1.9×10^5	1.4×10^2

Figure 2. Effect of splice donor point mutations on expression of a spliced gene. The *hph* gene (*hygro*) from pHygro Bgl II was inserted into the splicing vector prZNSV(X). Expression of *hygro* in prZNSV(X) hygro would be directed by non spliced mRNA while neo expression arrise from translation of spliced mRNA. To create prZNSV(X) 194 hygro the 194 splice donor mutation was cloned in place of the excised wild type splice donor of Mo MuLV (as a 'Kpn I to BamH I fragment) in prZNSV(X) hygro. Both constructs were transfected into PA317 cells and the transiently produced virus used to infect Ψ-2 cells, which were hygromycin B selected to establish stable ecotropic producer lines. The supernatants of the stable producer populations were used to infect NIH 3T3 cells and both hygro^r and G418^r titres were quantitated. The results shown represent the average of two independent experiments.

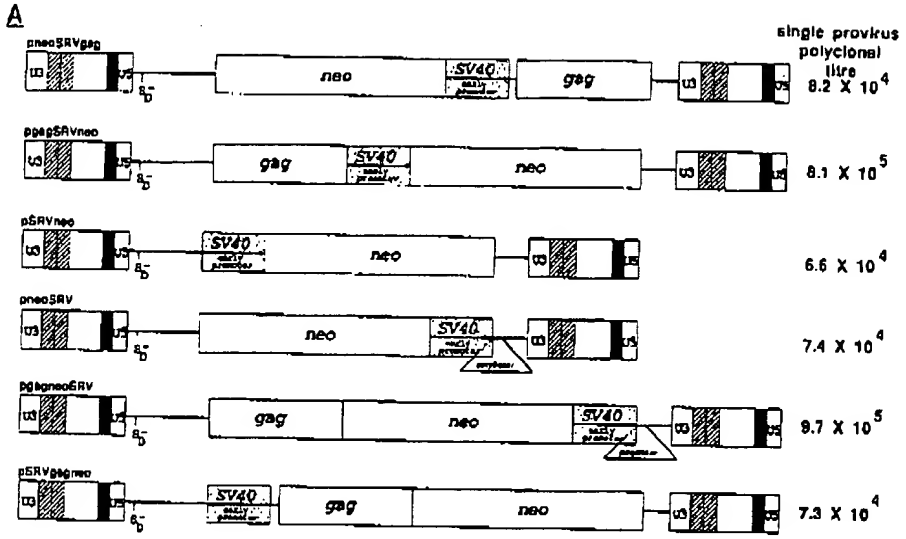
hygro^r titre derived from non-spliced mRNA was equivalent for both vectors.

Effect of *gag* Sequences on Vector Titre

With the use of the 194 splice donor mutant DO constructs with titres nearly as high as those of vectors with wild type splice donors such as pZipNeoSV(X) could be obtained. However, wild type Mo MuLV produces titres in excess of 10^6 pfu/ml in contrast to the aforementioned recombinant vector titres of 10^5 cfu/ml. On the other hand, the N2 vector, which was unique in its maintenance of *gag* coding sequences, possessed the capactiy to transmit a *neo*^{r} gene at titres tenfold higher than most other existing retroviral vectors (9).

As a direct test of their effect on vector titre, *gag* sequences were inserted into a conventional *gag*$^-$ DO vector, pneoSRV. Fusion of the *gag* sequences in frame with the *neo* gene produced the *gag*$^+$ construct pgagneoSRV. Two indepentent experiments measuring the respective virus titres revealed that indeed *gag* sequences can potentiate recombinant vector titre by an order of magnitude (see Figure 3A for averaged results).

To further explore the mechanism by which *gag* sequences exert their effect on vector titre, four additional vectors in which the *gagneo* fusion was separated into its constituent parts or expressed from the internal SV40 promoter were assembled, and two independent titrations performed. The results in Figure 3A can be summarised as follows: To attain increased recombinant vector titre, *gag* sequences must be inserted at their native position within wild type virus, adjacent to the Ψ-site, but not necessarily as a *gagneo* fusion. The high titres associated with *gag*$^+$ vectors were not due to helper virus since XC assays (23) of the Ψ-2 producers of the six vectors proved negative. Northern blot analysis of the vector derived mRNAs present in polyclonal Ψ-2



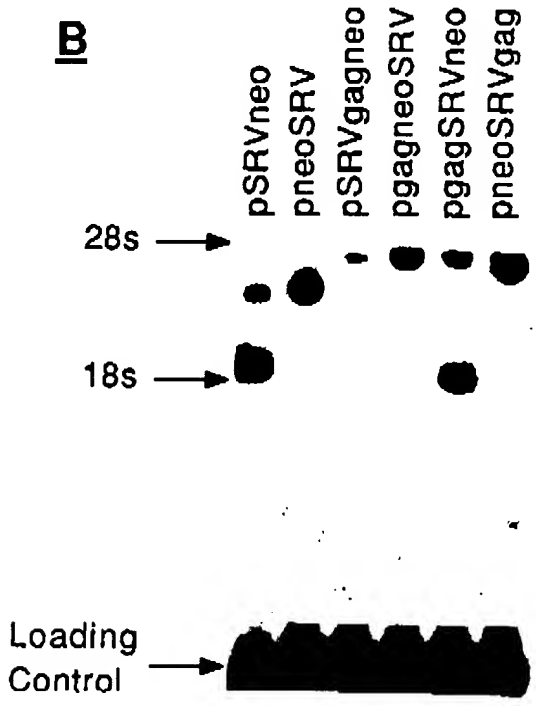


Figure 3. Effects of *gag* sequences on vector titre. A) Titration of pSRV derived constructs. Six constructs derived from the vector pSRV with *gag* sequences from a proviral clone of Ableson MuLV (44) in varying positions were assembled. Each construct was transfected into PA317 cells and the transiently produced virus used to infect Ψ-2 cells, which were G418 selected to establish stable ecotropic producer lines. The supernatants of the six stable producer populations were used to infect NIH 3T3 cells and the G418^r titres were quantitated. The results shown represent the average of two independent experiments. B) Northern Analysis of mRNA in stable Ψ-2 producer populations. Total RNA was prepared from each of the six Ψ-2 producer cell populations, and 10 μg of each sample electrophoresed through a formaldehyde/1% agarose gel. The gel was blotted onto a GeneScreen Plus membrane membrane and hybridised to ^{32}P-labelled probe made from a 1 kb Bgl II fragment containing the *neo* gene of Tn*5*. The blot was stripped by boiling and re-hybridised to a ^{32}P-labelled γ-actin probe to normalise for amounts of RNA loaded/transferred onto the blot.

producer cell lines used in the above titrations shown in Figure 3B demonstrates that *gag* sequences did not augment vector titre by increasing steady state levels of genomic vector RNA. Specifically, more genomic RNA was found in the pneoSRVgag producer population than the producer population harbouring the reciprocal construct pgagSRVneo, yet the titres associated with the latter producer cell line were ten-fold higher.

Effect of *env* Sequences on Vector Titre

Since *gag* coding sequences at the 5′ end of retroviral vectors had been shown to enhance vector titre, the contribution of

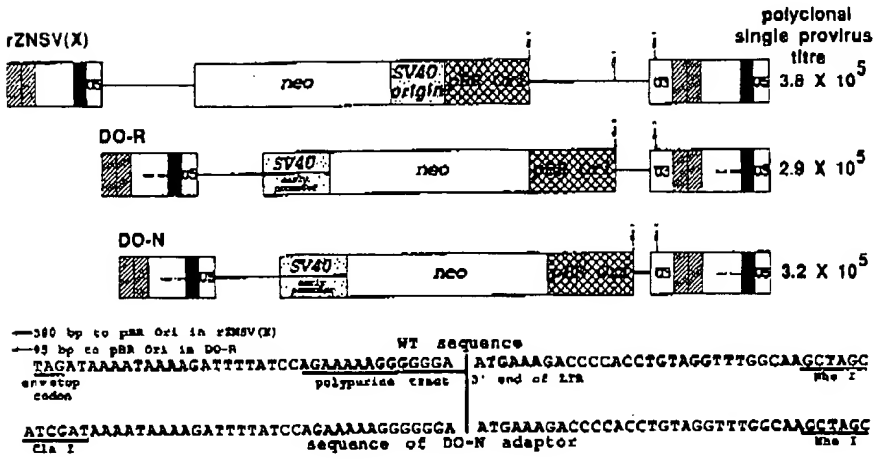


Figure 4. Effects of *env* sequences on vector titre. An adaptor composed of synthetic oligonucleotides was inserted in place of the excised Cla I to Nhe I fragment of pDO-R to delete its 95 bp of *env* coding sequences. The resultant construct, pDO-N, and two control vectors bearing different amounts of *env* coding sequences, prZNSV(X) and pDO-R, were transfected into PA317 cells and the transiently produced virus used to infect Ψ-2 cells, which were G418 selected to establish stable ecotropic producer lines. The supernatants of the stable producer populations were used to infect NIH 3T3 cells and the $G418^r$ titres were quantitated. The results shown represent the average of two independent experiments.

sequences at the 3′ end towards vector titre was explored. It was of considerable interest to determine whether *env* coding sequences retained in all retroviral vectors were dispensable in a high titre retroviral vector. This point had become relevant in light of findings that vectors that retained the most wild type sequences, such as N2, were more likely to generate wild type virus as they were passaged in packaging cells, presumably via homologous recombination events (11). As a consequence, all *env* coding sequences 5′ of the *env* stop codon were deleted from the vector pDO-R yielding pDO-N. Supernatants of stable Ψ-2 producer populations of pDO-N, pDO-R and pZipNeo SV(X) (which contain 0 bp, 95 bp, and 580 bp of *env* coding sequences respectively) were titrated on NIH 3T3 cells. No decrease in titre was observed for the pDO-N vector relative to the *env*+ vectors as shown in Figure 4, indicating that *env* coding sequences are not required in *cis* during the Mo MuLV life cycle.

pBabe Vectors

Initial attempts to utilise pgagneoSRV (Figure 3A) as an expression vector invariably led to limited or non detectable expression of several genes inserted 3′ of the vector's internal SV40 early promoter. Substitution of the rat β actin or simian cytomegalovirus immeadiate early (CMV IE94) promoter for the SV40 early promoter in pgagneoSRV did not improve the vector's ability to express exogenous genes (data not shown). Because of these failures, an alternative approach that would utilise the strong promoter in the LTR for expression of an inserted gene while still maintaining *gag* sequences was sought.

In order to augment vector titre *gag* sequences must be located at the 5′ end of the vector next to the Ψ-site (see Figure 3A). However, at this position the initiator ATG of *gag* is the first ATG within a favorable context for translation initiation, precluding translation of genes inserted 3′ of these *gag* sequences (24). Therefore, a mutagenised *gag* cassette, lacking the normal initiator codon, from the vector pLNL 6 (25) was incorporated into two new DO vectors with expanded polylinkers but without functional splice donors and *env* coding sequences. The resultant high titre 'pBabe' vectors were designed to express inserted genes from the promoter within the LTR and an mRNA encoding drug resistance markers from the internal SV40 early region promoter (Figure 5). pBabe Neo and pBabe Hygro confer resistance to kanamycin/G418 or hygromycin B in procaryotes and eucaryotes, respectively, allowing quick recovery of integrated proviruses derived from these vectors as plasmids by 'shuttling' them into bacteria (1).

To quantitatively determine if the ATG^- *gag* casette in the pBabe vectors would confer high titre characteristics to the vectors without inhibiting translation of inserted genes, the *cat* and v-Ha-*ras* genes were cloned into both vectors and their expression levels compared to those of these genes cloned into a conventional DO vector lacking *gag* sequences.

In all cases, pBabe vector titres were greater than 10^6 cfu/ml, exceeding conventional gag^- DO vector titres by at least an order of magnitude. No significant difference in stable *cat* activity was noted between the conventional gag^- vector DO-R cat and pBabe Neo cat (Figure 6). Similarly, (cfu and ffu) titrations of stable producers of DO-R ras and pBabe Neo ras or pBabe Hygro ras on NIH 3T3 cells resulted in identical ratios of drug resistant colonies to morphologically transformed foci for both the gag^- and ATG^- gag^+ vectors (Figure 7A). Examination of the $p21^{v\text{-}Ha\text{-}ras}$ protein levels in drug resistant NIH 3T3 cells resulting from infection with the above vectors revealed no significant difference in the amount of $p21^{v\text{-}Ha\text{-}ras}$ produced by the gag^- and ATG^- gag^+ vectors (Figure 7B). Last, no aberrant electrophoretic mobility of the $p21^{v\text{-}Ha\text{-}ras}$ produced by the ATG^- gag^+ pBabe vectors was observed, indicating the expressed $p21^{v\text{-}Ha\text{-}ras}$ was not a fusion protein resulting from initiation at an upstream *gag* ATG codon.

To expand the range of available drug selection schemes, two additional pBabe vectors with different drug selection markers were constructed. pBabe Bleo allows growth of infected cells in the presence of phleomycin, while pBabe Puro permits selection in medium containing puromycin (Figure 5). Both vectors maintain the high titre characteristic of the other pBabe vectors (J. P. Morgenstern, Ph. D. thesis), but are not shuttle vectors as they are not capable of conferring resistance in bacteria.

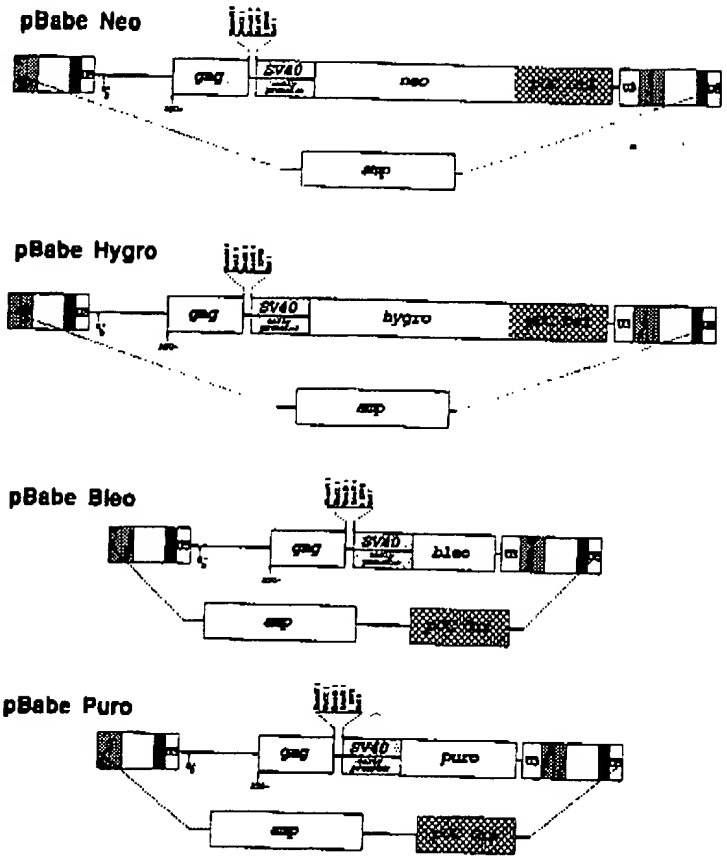
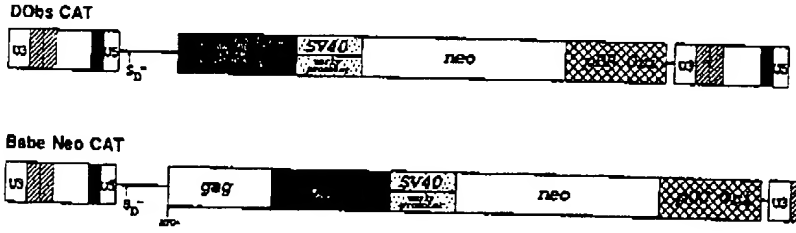


Figure 5. Schematic representation of pBabe vectors each containing a different selectable marker gene. Drug resistance genes were derived from the following plasmids: *neo*, pSV2 Neo (10), *hph*, pHygro Bgl II, *ble*, pUT 714 (45), and *pac*, pSV2 pac (46). The hatched boxes represent the 72 bp repeats of the enhancer and the blackened boxes the R region beteween the transcriptional start and the polyadenylation sites within the Mo MuLV LTRs.

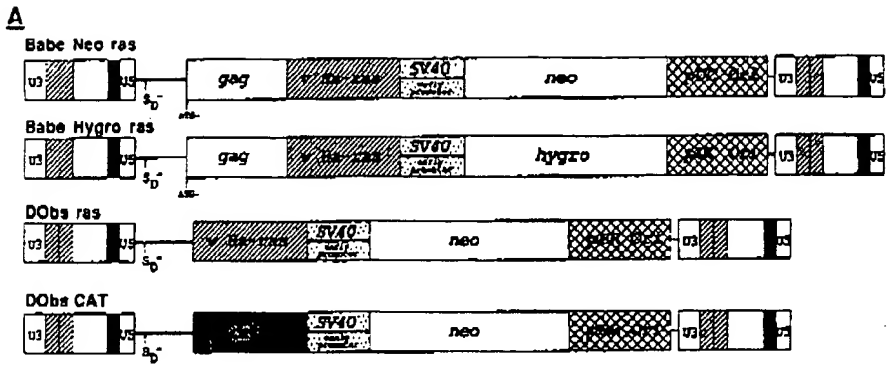


Construct	single provirus polyclonal (cfu/ml) G418^r titre	psi-2 single provirus polyclonal (cpm) cat activity	NIH 3T3 single provirus polyclonal (cpm) cat activity
DObsCAT	3.6×10^5	2.4×10^4	2.8×10^4
Babe Neo CAT	5.5×10^6	2.7×10^4	2.6×10^4

Figure 6. Comparison of titre and expression of a bacterial reporter gene by pBabe Neo and pDO-R. The *cat* gene from Tn9 was inserted into pBabe Neo and pDO-R. Both constructs were transfected into PA317 cells and the transiently produced virus used to infect Ψ-2 cells, which were G418 selected to establish stable ecotropic producer lines. The supernatants of the stable producer populations were used to infect NIH 3T3 cells and the G418^r titres were quantitated. The results shown represent the average of two independent experiments. The enzymatic CAT activity present in both the Ψ-2 stable producer populations and populations of NIH 3T3 cells pooled from the G418 titrations were assayed and normalised for protein content. The results shown are the average of two CAT assays on each cell population.

ΩE: a High Titre Third Generation Ecotropic Helper-Free Packaging Cell Line

As previously noted, wild type virus is much more likely to arise when passaging the *gag*$^+$ vector N2 in helper-free packaging cell lines relative to *gag*$^-$ vectors (11). The provirus-like packaging construct lacking Ψ (packaging) sites present in 'first generation' packaging cell lines, such as Ψ-2 cells, could be restored to a wild type provirus by a single recombination event,



Construct	G418^r cfu/ml	hygro^r cfu/ml	ffu/ml
Babe Neo ras	4.4×10^5	0	4.1×10^5
Babe Hygro ras	0	2.5×10^5	2.7×10^5
DO-R ras	2.5×10^4	0	2.3×10^4
DO-R cat	2.6×10^5	0	0

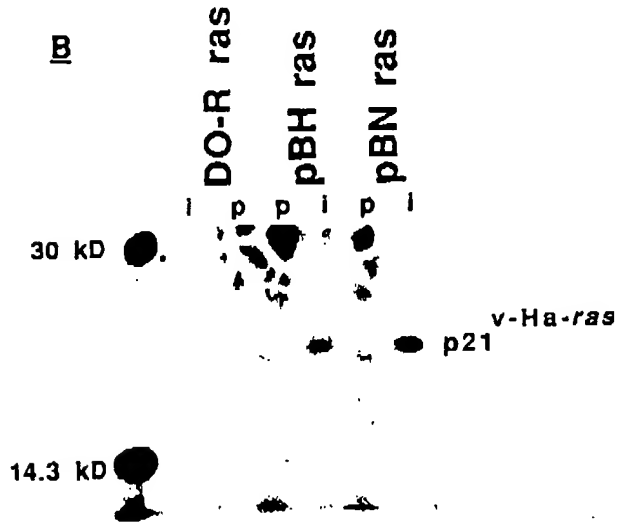


Figure 7. Comparison of titre and expression of a viral oncogene by pBabe Neo/pBabe Hygro and pDO-R. A) The v-Ha-*ras* gene of Ha MuSV was BamH I linkered and inserted into pBabe Neo, pBabe Hygro and pDO-R. All three constructs transfected into Ψ-2 cells and the resultant G418^r or hygromycin B^r colonies pooled as stable producer populations. The supernatants of the stable producer populations were used to infect NIH 3T3 cells and the G418^r, hygromycin B^r and morphological focus forming titres were quantitated. Supernatants of Ψ-2 producers of pDO-R cat were included as a negative control for focus forming ability. B) The amount of p21$^{v\text{-}Ha\text{-}ras}$ present in pooled G418^r or hygromycin B^r NIH 3T3 cell populations infected with pBN ras, pBH ras or pDO-R ras was examined by immunoprecipitation/SDS PAGE analysis. p: pre-immune rat serum as the primary antibody; i: rat monoclonal α p21$^{v\text{-}Ha\text{-}ras}$ as the primary antibody.

apparently enhanced by the addition of homologous *gag* sequences in N2. This led investigators to generate the 'second generation' helper free packaging cell line PA317 whose packaging construct lacks both a Ψ-site and a 3′ LTR, necessitating two recombination events with a vector to restore a wild type proviral structure (16). Although for the most part helper virus is rarely observed in PA317 cells, it still has been detected (26). By placing the the *gagpol* and *env* reading frames on separate (Ψ site and 3′ LTR minus) constructs 'third generation' cell lines (27–29) restrict the generation of wild type virus to three separate recombination events.

To further reduce the probability of the aforementioned three recombination events occurring during the passaging of the *gag*$^+$pBabe vectors, an improved third generation ecotropic packaging cell line was constructed (Figure 8A). First, the region of overlap between the Ψ-site/3′ LTR minus *gagpol* and *env* expression constructs was reduced to the minimum of 61 bp (found in different reading frames of the *pol* and *env* genes of Mo MuLV) in an effort to decrease the possibility that they would recombine to form a contiguous *gag/pol/env* product (Figure 8B). Transversion mutations were introduced into at least the third base of each of the final 20 codons of the *pol* gene in the construct pJ4Ω 5′ WT gagpol. This 'codon wobbling' maintains the primary protein sequence of the integrase region of the *pol* open reading

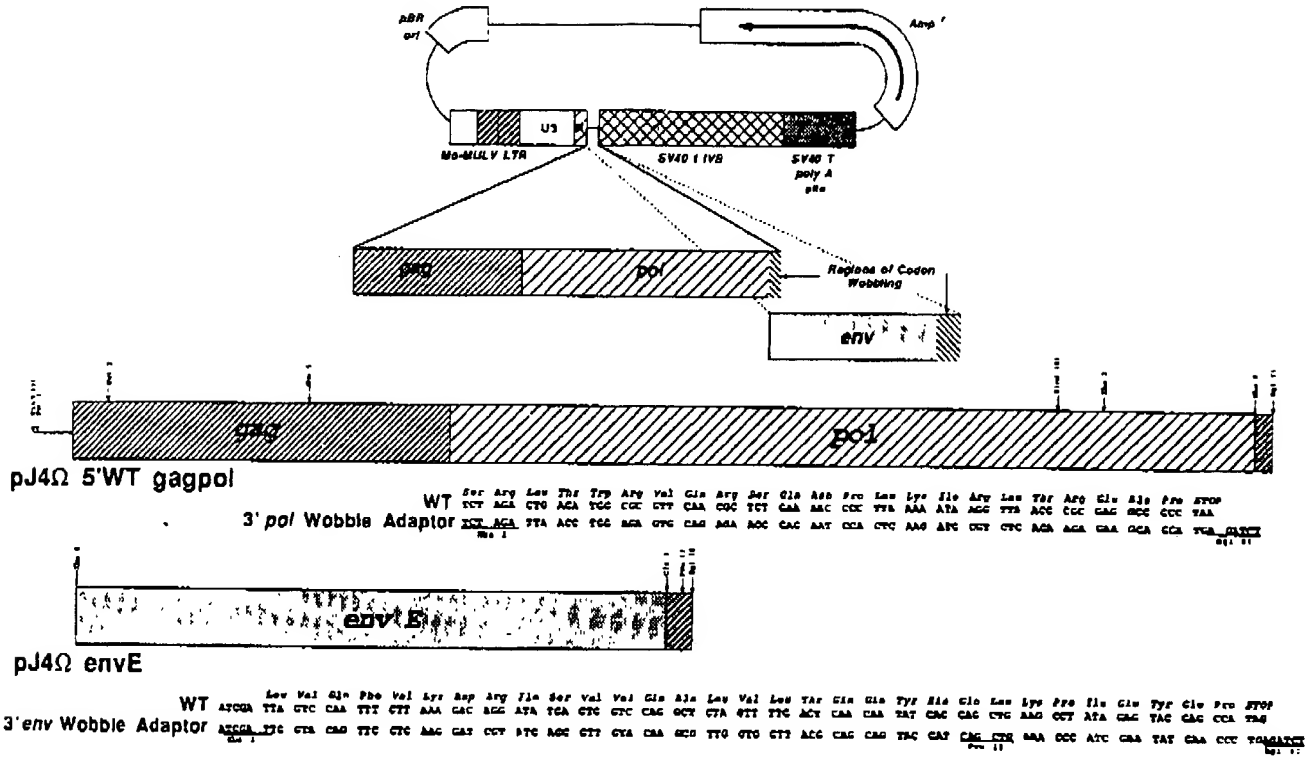


Figure 8. Schematic representation of ΩE third generation packaging *gagpol* and *env* constructs in pJ4Ω (47). The exact derivation of pJ4Ω gagpol and pJ4Ω envE, including a comparison of wild type Mo MuLV coding sequence and codon 'wobble' adaptors used to decrease nucleotide sequence homology between the constructs is indicated. The wobble adaptors were subcloned into pJ4Ω, after which the *gagpol* orf was inserted as a fragment from a Pst I to Xba I partial digest of the NB-tropic proviral clone pMov 9-2 (48) (positions 739 to 5325 of Mo MuLV) and the ecotropic *env* coding region was inserted as an Xba I to Cla fragment (positions 5766 to 7674 of pMo Mu LV).

frame (orf), but decreases the primary nucleotide sequence homology over the 61 bp region of *pol/env* overlap in pJ4Ω 5' WT gagpol and pJ4Ω envE to 55%. At the 3' end of the ecotropic *env* expression construct pJ4Ω envE the final 31 codons were wobbled (decreasing homology with the ecotropic Mo MuLV to 62%), and all sequences 3' of the *env* stop codon (polypurine tract and 3' LTR) were deleted (Figure 8B).

Both constructs were co-transfected with the pSV2 gpt marker (30) into NIH 3T3 cells, and 14 days later 192 mycophenolic acid resistant colonies were picked and assayed for reverse transcriptase activity in their supernatants (31). Twelve clones with the highest reverse transcriptase levels (as well as Ψ-2 cells as a positive control) were infected with an amphotropic stock of pBN cat. Subsequently, G418 resistant colonies were pooled, and their supernatants used in titrations on NIH 3T3 cells. The above procedure was repeated for the three packaging clones yielding the highest titres in the intial experiment. Clone 2E1, which gave titres 50% as high as Ψ-2 (15) or the 'third generation' GP+E (27) packaging cells, was designated as the ΩE packaging cell line.

As a preliminary test of the ΩE cell line's propensity for generating wild type virus, a polyclonal population of ΩE cells with pBN cat proviruses from the above titrations were passaged for four weeks and their undiluted supernatant used to infect 7.5×10^5 NIH 3T3 cells. In order to promote any potential helper virus to spread, the infected NIH 3T3 cells were not selected in G418 and were passaged for two weeks in the presence of 2 μg/ml of polybrene. Undiluted supernatant from the infected/passaged NIH 3T3 cells was used to infect 7.5×10^5 fresh NIH 3T3 cells, all of which were split and selected in G418. After 14 days no $G418^r$ colonies, indicative of contaminating helper virus in the initial ΩE pBN cat producer supernatants, were detected.

DISCUSSION

By exploring the role of coding sequences of the Mo MuLV genome as *cis* acting signals in the retroviral life cycle, we have been able to develop a highly efficient retroviral based gene transfer system for stable expression within mammalian cells. While a number of laboratories have reported either high titre retroviral vectors or safe helper free packaging cell lines, we have extended their initial observations and designed a series of highly efficient vectors in conjunction with a packaging cell line.

Initial work with DO retroviral vectors revealed that alterations in the splice donor region of Mo MuLV can have deleterious effects on vector titre. A similar effect was noted by another group, however they failed to observe a significant effect of *gag* sequences on vector titre (32). In order to avoid splicing artifacts, a splice donor point mutant which did not adversely affect vector titre was made and demonstrated to inhibit splicing (as infered by a 10^3-fold decrease in $G418^r$ titre) when introduced into the vector pZipNeo SV(X). A similar inhibition of splicing was obtained when the identical splice donor mutation from Ha MuSV was introduced into the splicing vector N2 (33).

Work presented in this paper is also in agreement with results showing that *gag* sequences enhance retroviral vector titre (25, 34). These findings indicate that *gag* sequences achieve their effect by directly increasing the efficiency of packaging of RNA into budding virions, in *cis*, and without the need for their translation. In addition, our work shows that unlike the core region of the Ψ-packaging site (35), the *gag* sequence portion of the Ψ-site cannot be moved from its native position within the Mo MuLV genome and still maintain its activity.

Taken together, the effects of both the splice donor region and *gag* sequences on retroviral vector titre strongly argue that the packaging region of Mo MuLV extends both 5′ and 3′ of the previously arbitrarily defined Ψ-site (15). Hence the core sequences of the Ψ-site are necessary and sufficient for conferring the packaging function in *cis* to an RNA transcript (35, 36), while the flanking sequences modulate the efficiency of the packaging function. Indeed one investigation has even implicated sequences as far 5′ as the U5 region as being involved in Mo MuLV genome packaging (37). When simultaneously incorporated into the pBabe vectors, the 194 splice donor point mutation (2.3×10^5 cfu/ml for pLRV 194) and *gag* sequences (8.1×10^5 cfu/ml for pgagSRVneo) interact multiplicatively to yield stable vector titres in excess of 2×10^6.

Converse to the findings for *gag* sequences, deletion of all *env* coding sequences had no effect on Mo MuLV based retroviral vector titre, demonstrating the lack of any *cis* acting replication signals within the *env* gene. Exclusion of non essential *env* sequences from retroviral vectors should eliminate homologous recombination between such vectors and second and third generation packaging constructs (see discussion of packaging constructs below).

By incorporating the above findings into a single construct, the pBabe vectors basically fulfil the potential of the DO design for retroviral vectors, transmitting and expressing exogenous genes, subsequent to selection, at high levels. These vectors rely on the Mo MuLV LTR's promoter for transcription of inserted genes, which has proven to be the more efficient than most internal promoters in a number of cell types (38, 39). As yet there is no evidence that the ATG$^-$ *gag* sequences responsible for the high titre nature of the pBabe vectors will compromise expression of an inserted gene at the level of translation. Currently more than eight genes have been successfully expressed within the pBabe vectors or those of similar design (33, L. Penn, M. Brooks and H.L., unpublished). Therefore, it is likely that this lack of inhibition of translation by ATG$^-$ *gag* sequences is a general phenomenon and therefore an inherent characteristic of these vectors. No aberrant splicing interactions with inserted genes have been observed in our experience with any vector containing the 194 splice donor mutation incorporated in the pBabe vectors.

None of the resistance activities encoded in the *neo*, *hph*, *ble*, or *pac* genes of the pBabe vectors overlap, making it possible to introduce four different genes into a single cell. This capability could be of great use in the study of any number of biological phenomena involving the interaction of multiple genes. In this context it is also worth mentioning that the selection for *pac* expression with puromycin is the most powerful selection procedure. Moreover, because pBabe Neo and pBabe Hygro are shuttle vectors with inverted BstX I restriction sites for efficient cDNA cloning (40), they are candidate vectors for use in the construction of cDNA expression libraries for use in complementation assays in mammalian culture systems (J. P. Morgenstern, Ph. D. thesis, University College, London).

The pBabe vectors harbour limited potential to yield wild type virus via homologous recombination with defective proviral 'packaging' constructs in helper free packaging cell lines (11). In contrast to many N2 based *gag*$^+$ vectors that maintain intact 5′ proviral structures (41, 42), the Ψ-site in the pBabe vectors is flanked by both an attenuated splice donor and ATG$^-$ *gag* sequences. At the pBabe vectors' 3′ end, all *env* sequences have been deleted, confining recombination to sequences within the polypurine tract and 3′ LTR, neither of which are present in the packaging constructs of the PA317 or ΩE packaging cell lines. All other vectors, except those whose 3′ ends are derived from pBabe constructs (33), contain somewhere between 100 to 550 bp of p15E coding sequences.

The high titre ΩE cell line represents a third generation helper free packaging cell line designed specifically to prevent recombination between the packaging constructs themselves, and the pBabe retroviral vectors (see previous paragraph). The successful use of codon wobbling to alter nucleotide sequence but maintain coding information in the *gagpol* and *env* packaging constructs of the ΩE cell line indicates that this may be a generally applicable method for reducing the probability of recombination between two known homologous coding sequences. To enable introduction of the pBabe vectors into a wide variety of mammalian cells, an amphotropic analog the ΩE cell line is currently under construction.

During the construction of the ΩE packaging cell line, two other groups have published Mo MuLV based third generation packaging cell lines (27–29). Both ΨCRE/ΨCRIP and GP+E/GP + envAm12 use separate *gagpol* and *env* packaging constructs lacking Ψ-sites and LTRs at their 3′ ends. Sequential transfection with different selectable markers was used to transfer the two Ψ-CRE/CRIP and GP+envAm12 packaging constructs into cells, whereas the GP+E and ΩE constructs were cotransfected together with a single marker. Here there may be an advantage to sequential introduction given the high propensity of DNA molecules to recombine during calcium phosphate transfection (43), although no helper virus has yet been detected in the ΩE cells.

The Ψ$^-$ CRE/CRIP *gagpol* and *env* packaging constructs contain reciprocal linker insertion mutations that destroy the *gag* and *env* open reading frames, rather than the reciprocal deletions found in the GP+E/GP + envAm12 and ΩE packaging constructs. It is likely that the large (>4 kb) region of overlap in the *gagpol* region CRE/CRIP packaging constructs will be much more prone to participate in recombination than the relatively small overlap (500 bp) in the GP+E/GP + envAm12 constructs. Here, greater care has been taken to prevent recombination in the ΩE constructs, with only a 66 bp region of overlap, whose sequence homology has been reduced to 55% by codon wobbling.

At the 3′ end of each of the *env* packaging constructs of the three cell lines, only the pJ4Ω envE construct has been designed to eliminate all sequences 3′ to the *env* stop codon. In the two other *env* packaging constructs the polypurine tract, inverted repeat at the LTR boundary, and portions of the 3′ LTR are retained. The significance of overlap of these sequences with those found in retroviral vectors (other than *env*$^-$ vectors such as pBabe) became apparent when helper virus was detected during shuttling of recombinant virus between Ψ-CRE and PA317 cell lines. Recombination in this case appears to have occurred between the sequences 3′ to *env* in one of the Ψ$^-$CRE

packaging constructs and the retroviral vector (29). Although not formally proven, one may expect that the reduction of sequence overlap and homology in the Ω packaging constructs will probably aid in the reduction of the frequency of helper formation in the ΩE cell line relative to the other Mo MuLV based third generation packaging cell lines. •

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Retroviral Gene Transfer Using Safe and Efficient Packaging Cell Lines

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INTRODUCTION

The introduction of normal genes into the dividing cells of individuals affected by genetic disorders, termed gene therapy, has been the focus of much research over the past decade. Retroviruses appear to be the method of choice as vehicles to deliver exogenous genes into human cells. This is due to the high efficiency of retroviral gene transfer, as well as the fact that the retroviral proteins necessary to form the virion particle can be supplied in *trans*. The retroviral gene transfer system employs the use of two types of virus: a retroviral vector containing the gene to be transferred, and the helper or "packaging" virus which provides *gag, pol* and *env* proteins in *trans*. The vector virus contains the viral long terminal repeats (LTRs) and the packaging sequence but lacks the *gag, pol* and *env* gene sequences. The helper virus, contained in a packaging cell line (such as $\psi 2$[1]), has a deletion of the ψ packaging sequence, which is required in *cis* for the packaging of retroviral RNA into the virion.

An important prerequisite for the use of retroviruses for gene therapy is the availability of safe retrovirus packaging cell lines incapable of producing wild-type virus.[2] The major danger of the use of retroviral vectors for gene transfer is the possibility that replication-competent viruses could be generated through recombination events in which the intact ψ sequence from the vector virus corrects the deleted ψ sequence of the helper virus. The proliferation of wild-type virus can lead to multiple integrations into the genome, which may result in the activation of potentially harmful genes such as oncogenes.[3,4] Packaging cell lines containing additional mutations, including deletions of the 3′ LTR and portions of the 5′ LTR in the PA317 cell line,[5] have been constructed as safer alternatives to the $\psi 2$ cell line. When PA317 cells are used, two recombination events are necessary to form a wild-type genome. Nevertheless, results from several laboratories indicate that, even when several mutations are present, wild-type virus can still be generated using PA317 cells.[6,7]

We have approached the problem of creating safe and efficient packaging cell lines by separating the viral *gag, pol,* and *env* genes of the helper virus onto two

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plasmids; *gag* and *pol* are on one plasmid and *env* is on the other. In addition, the ψ packaging sequence and the 3′ LTR have been removed in both plasmids. When the resulting ecotropic (GP+E-86) and amphotropic (GP+*env*Am12) packaging lines are used, at least three recombination events between the helper virus genome and the vector virus genome are necessary to generate a wild-type virus. Generation of replication-competent virus has not been detected with these packaging cell lines, and their efficiency of gene transfer is comparable to that of packaging lines containing the viral genes on one plasmid. These cell lines have also been demonstrated to be successful for use in gene transfer into live mice.

RESULTS

Generation of the Ecotropic Packaging Line

To generate the ecotropic packaging cell line, two helper virus plasmids, p*gag-pol*gpt[8] and p*env*[8] were constructed using Moloney murine leukemia virus (Mo-MULV) proviral DNA from the plasmid 3PO[9] (FIG. 1). The plasmid p*gag-pol*gpt was

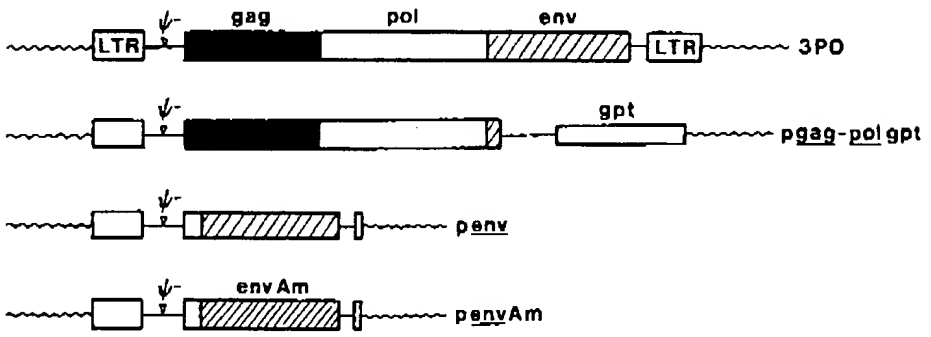

FIGURE 1. Comparison of viral sequences contained in plasmid 3PO and constructs p*gag-pol*gpt, p*env*, and p*env*Am. Mo-MULV LTRs and the ψ deletion (ψ⁻) are indicated. *Solid boxes* represent *gag* sequences; *open boxes, pol* sequences; *hatched boxes, env* sequences; *wavy lines,* pBR322 sequences.

constructed by isolating a fragment containing the 5′ LTR and the *gag* and *pol* DNA from 3PO and inserting this fragment into the plasmid pSV2gpt.[10] The plasmid p*env* was constructed by isolating a fragment from 3PO that contains the 3′ acceptor splice site and the *env* gene and ligating it to another fragment from 3PO containing the 5′ LTR and 5′ donor splice site.

3T3 cells were transfected by electroporation with p*gag-pol*gpt and p*env* DNAs, and recipient cells were selected for the presence of the *gpt* gene with media containing mycophenolic acid (MA).[8] MA-resistant (GP+E) clones were isolated, and their supernatants were tested for reverse transcriptase (RT) activity.[8,11] GP+E clones which produced high levels of RT were then tested for *env* protein production by immunoprecipitating labeled cellular proteins with anti-*env* antisera.[8]

Five GP+E cell lines which expressed high levels of RT and *env* proteins were tested for their ability to package the Δneo retroviral vector[8] (FIG. 2) These cell lines were transfected with Δneo DNA. G418-resistant clones were isolated and tested for their release of *neo* gene–containing viral particles by using harvested supernatants

N2 -

Δneo —

FIGURE 2. *line,* pBR32 site; SA, 3′ :

to infect (2.0 × 10² than those data not packaging pSV2gpt i

TABLE 1. V

Packa
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3PO-
GP+1
3PO-

[a]Represe
[b]CFU, col

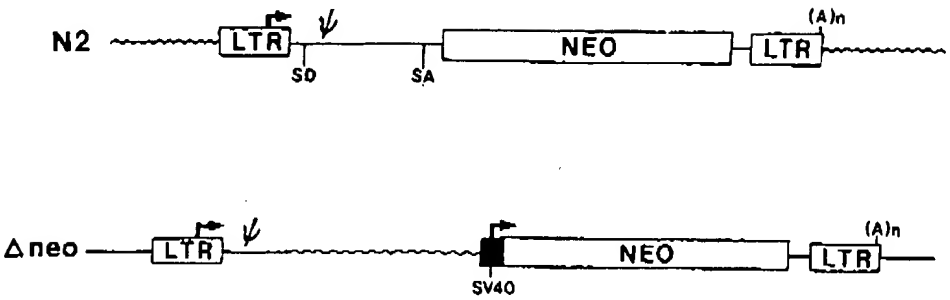

FIGURE 2. Replication-defective retroviral vectors N2 and Δneo. ψ, packaging sequence; *wavy line*, pBR322 sequences; *solid box*, SV40 promoter and origin of replication. SD, 5′ splice donor site; SA, 3′ splice acceptor site.

to infect 3T3 cells.[8] The GP+E-86 packaging cell line produced titers of Δneo (2.0×10^2 to 6.5×10^5 colony-forming units [CFU]/ml) that were consistently higher than those produced by any of the other four GP+E lines (TABLE 1 and additional data not shown). Titers were comparable to those obtained from the 3PO-18 packaging line,[8] which was generated by co-transfecting the plasmids 3PO and pSV2gpt into 3T3 cells.

TABLE 1. Virus Production From Ecotropic Packaging Cell Lines

Packaging Line	Vector	Clone[a]	Titer (CFU/ml)[b]
GP+E-86	Δneo	1	1.2×10^5
		8	3.0×10^4
		11	9.0×10^4
		17	6.5×10^4
		21	1.7×10^5
3PO-18	Δneo	1	2.2×10^4
		2	1.1×10^4
		3	5.7×10^3
		4	8.0×10^2
		5	6.7×10^3
GP+E-86	N2	3	1.2×10^6
		7	3.5×10^6
		8	2.6×10^6
		9	3.0×10^6
		11	1.3×10^6
		12	4.0×10^6
		13	3.6×10^6
3PO-18	N2	1	3.8×10^4
		2	1.8×10^4
		3	4.0×10^4
		6	1.0×10^5
		9	6.0×10^4
		10	1.0×10^5
		11	1.7×10^5

[a]Representative clones are shown.
[b]CFU, colony-forming units.

GP+E-86 cells were also tested for their ability to package the N2 retroviral vector[12] (FIG. 2). N2 DNA was transfected into GP+E-86 cells, and G418-resistant clones were isolated. Supernatants were then used to infect 3T3 cells. Titers of N2 virus ranged from 5.3×10^3 to 4.0×10^6 CFU/ml (TABLE 1). These titers were also comparable to those obtained using the 3PO-18 packaging line.

Generation of the Amphotropic Packaging Line

In order to create a packaging line to transfer retroviral vectors into human and primate cells, we substituted a plasmid containing an amphotropic *env* gene, p*env*Am,[13] for the ecotropic *env* gene in p*env*. The plasmid p*env*Am was constructed using DNA from the plasmid pL1,[14] which contains the 4070A amphotropic murine leukemia virus proviral DNA.[15] A fragment containing the *env* gene and the 3′ acceptor splice site was isolated and ligated to a fragment from 3PO containing the Mo-MULV 5′ LTR and 5′ donor splice site. The amphotropic packaging line was generated by first transfecting the p*gag-pol*gpt plasmid into 3T3 cells and selecting a MA-resistant clone, GP101, that produced a high level of RT.[13] The plasmids p*env*Am and pRSVhyg[16] were then co-transfected into GP101 cells. Clones resistant to 200 μg/ml hygromycin B[17] were isolated and tested for amphotropic *env* protein production by metabolic labeling followed by immunoprecipitation with anti-*env* antiserum.[13] The clone GP+*env*Am12 was selected for use because it was the cell line which produced a significantly higher level of amphotropic *env* protein than the other clones tested.

To test for packaging ability, GP+*env*Am12 cells were transfected with the N2 retroviral vector. G418-resistant clones were isolated, and titers of released N2 virus were determined by infecting 3T3 cells with harvested supernatants. The N2 viral titer produced by the GP+*env*Am12+N2 clones ranged from $< 10^2$ to $> 10^6$ CFU/ml (TABLE 2). In a control experiment, N2 was transfected into the PA317 amphotropic packaging cell line. Titers of G418-resistant clones, when used to infect 3T3 cells, ranged from $< 10^2$ to 3.3×10^5 CFU/ml. A number of N2-transfected GP+*env*Am12 clones were also tested for their amphotropic packaging ability by using super-

TABLE 2. N2 Virus Production From Amphotropic Packaging Cell Lines

Packaging Line	Clone	Titer (CFU/ml)[a]		
		3T3	K562	HeLa
GP+*env*Am12	3	2.6×10^5	6.0×10^3	3.0×10^4
	4	2.0×10^5	4.0×10^3	1.3×10^4
	6	1.7×10^5	1.0×10^3	3.5×10^4
	11	1.0×10^6	5.3×10^3	2.7×10^5
	12	1.0×10^5	1.0×10^2	5.4×10^4
	16	3.0×10^5	5.0×10^3	5.1×10^4
	Pool[b]	1.0×10^6	6.4×10^4	5.6×10^3
PA317	4	5.3×10^4	1.0×10^3	1.0×10^3
	5	3.6×10^4	1.4×10^4	1.0×10^3
	6	1.0×10^4	6.0×10^2	3.0×10^2
	9	2.0×10^4	1.0×10^4	1.7×10^3
	10	3.3×10^5	3.6×10^3	2.5×10^3
	Pool[b]	8.0×10^3	$< 10^2$	8.0×10^2

[a]Titers were determined on three different cell types, as indicated.
[b]Pools represent > 100 clones.

TABLE 3. Virus Production from GP+E-86 Cells Infected with N2 (Transfect-Infect Method)

Clone	Titer (CFU/ml)
1	3.3×10^6
2	7.9×10^6
3	3.2×10^6
4	7.2×10^6
5	1.4×10^7
6	1.0×10^7
7	1.4×10^7
8	4.2×10^6
9	6.6×10^6
10	9.1×10^6

natants to infect human K562[18] and HeLa[19] cells. Titers on K562 cells ranged from 1.0×10^2 to 6.4×10^4 CFU/ml. Titers on HeLa cells ranged from 5.6×10^3 to 2.7×10^5 CFU/ml. These titers are comparable to those obtained with supernatants from PA317 cells.

High-titer retroviral producer cells were also generated by the use of both the GP+E-86 ecotropic packaging cells and the GP+*env*Am12 packaging cells. The N2 retroviral vector was transfected into GP+*env*Am12 cells by electroporation, and supernatant from pooled clones was used to infect GP+E-86 cells. The resulting "transfect-infect" clones produced titers as high as 1.4×10^7 CFU/ml when assayed on NIH 3T3 cells (TABLE 3).

Analysis for Recombinant Infectious Retrovirus

A provirus mobilization assay was utilized as a stringent test for replication-competent retrovirus which may have been generated through three recombination events between the helper genomes in the GP+E-86 and GP+*env*Am12 cell lines and the N2 vector. The provirus mobilization assay was designed to detect a transfer of replication-competent helper virus or packaging function. Supernatants from the packaging lines and from packaging lines producing N2 virus were used to infect 3T3 cells harboring the N2 provirus (3T3:N2 cells) and 3T3 cells harboring a *his* provirus, which allows histidine-independent cell growth (3T3:116 cells). If the supernatants used in these infections contained replication-competent helper virus, the infected 3T3:N2 cells or 3T3:116 cells would begin to secrete N2 or *his* virus particles. The infected cells were tested for N2 or *his* virus production by using harvested supernatants to infect fresh 3T3 cells; this was followed by selection with 800 μg/ml G418 (for cells infected with N2) or with 1 mg/ml L-histidinol (for cells infected with *his*). Results from provirus mobilization assays indicated that neither the GP+E-86 and GP+*env*Am12 packaging lines nor these packaging lines containing the N2 retroviral vector produce replication-competent helper virus (TABLE 4). Provirus mobilization occurred only when supernatants from 3T3 cells producing wild-type retrovirus (3T3:NCA cells) were used to infect 3T3:N2 cells or 3T3:116 cells.

In another type of long-term safety analysis, GP+E-86 cells and GP+*env*Am12 cells were transfected with N2, and pools containing 500–1000 clones were collected. Supernatants from the GP+E-86 pools were used to infect 3T3 cells, and supernatants from the GP+*env*Am12 pools were used to infect 3T3, K562 and HcLa cells.

TABLE 4. Provirus Mobilization Assay for Helper Virus Production

Cells Tested	N2 Provirus Mobilization	*his* Provirus Mobilization
GP+E-86	–	–
GP+E-86 + N2 clone 12	–	–
GP+E-86 + N2 pools	–	–
GP+*env*Am12	–	–
GP+*env*Am12 + N2 clone 11	–	–
GP+*env*Am12 + N2 pools	–	–
3T3	–	–
HeLa	–	–
K562	–	–
3T3:NCA	+	+

The infected cells were passaged for one month without G418 selection, to allow for the possible spread of a rare recombinant wild-type virus throughout the cell population. Supernatants from these infected cells (secondary supernatants) were then harvested and analyzed for wild-type virus by testing for RT activity and also by infecting 3T3 and HeLa cells and testing for G418-resistant cells. All of these supernatants were RT-negative and did not confer G418 resistance upon reinfection of 3T3 or HeLa cells (TABLE 5).

Transfer of the Neo^R *Gene into Irradiated Mice*

The *neo*^{R} (neomycin-resistance) gene of the N2 retroviral vector was transferred into irradiated mice to determine if the GP+E-86 and GP+*env*Am12 packaging lines are capable of transferring an exogenous gene into hematopoietic cells. Retroviral gene transfer and bone marrow transplantation were performed as described by Hesdorffer *et al.*[20] Marrow was harvested from the hind limbs of donor C57BL/6J mice which had received 48 h of treatment with 5-fluorouracil (500 mg/kg). The bone marrow was infected with N2 virus by coculturing with GP+E-86 high-titer producer cells (4×10^6–1.4×10^7 CFU/ml) for 48 h and then was selected in 2 mg/ml G418 for 24 h. After selection in G418, 1×10^6 nucleated cells were injected into the tail veins of irradiated recipient mice.

Transplanted mice were sacrificed at various time points, ranging from 12 days to 310 days post-transplantation. DNA was extracted from spleen colonies (from 12-day post-transplant mice), whole spleens, and bone marrow.[20] DNA samples were

TABLE 5. Long-Term Assay for Detection of Wild-Type Retrovirus

Cells Tested	Cells Infected	Cells Infected with Secondary Supernatant	G418-resistant Cells	RT
GP+*env*Am12 + N2 clone 11	3T3	3T3	–	–
GP+*env*Am12 + N2 pools	3T3	3T3, HeLa	–	–
GP+*env*Am12 + N2 pools	HeLa	3T3, HeLa	–	–
GP+*env*Am12 + N2 pools	K562	3T3, HeLa	–	–
PA317 + N2 pools	3T3	3T3, HeLa	–	–
GP+E-86 + N2 pools	3T3	3T3, HeLa	–	–

digested with appropriate restriction endonucleases and analyzed by Southern blotting of agarose gels using a 1.5-kilobase (kb) *neo*^{R} fragment as a probe.

The results of Southern blot analysis of spleen and marrow DNA digested with *Eco*R I (which cuts the N2 provirus at either end of the *neo*^{R} gene) indicate the presence of the *neo*^{R} gene between day 12 and day 200 following transplantation. The efficiency of gene transfer into irradiated mice ranged from 48% to 52% in the transplanted mice (11/17 mice in one experiment and 6/11 mice in another experiment). Whole spleen DNAs from these mice were also digested with *Hind* III, which does not cut within the N2 provirus, to determine the number of viral integration sites per transplanted mouse. The number of viral integration sites reflects the number of infected hematopoietic stem cells that repopulated the transplanted mouse, assuming one viral integration per stem cell. Between three and six integration sites were apparent in the spleen DNAs from transplanted mice.

SUMMARY

One of the requirements for the use of retroviral vectors in human gene therapy is a packaging cell line which is incapable of producing replication-competent virus and which produces high titers of replication-deficient vector virus. Wild-type virus may be produced through recombinational events between the helper virus and a retroviral vector. We have constructed an ecotropic packaging cell line, GP+E-86, and an amphotropic packaging cell line, GP+*env*Am12, in which the viral *gag* and *pol* genes are on one plasmid and the viral *env* gene is on another plasmid. Both plasmids contain deletions of the packaging sequence and the 3′ LTR. The fragmented helper virus genomes, when introduced into 3T3 cells, produce titers of retrovirus which are comparable to the titers produced from packaging cells containing the helper virus genome on a single plasmid. We have found no evidence for the generation of wild-type retrovirus using the GP+E-86 and GP+*env*Am12 packaging lines, either alone or in combination with the N2 retroviral vector. We also show that these packaging cell lines can be used to transfer the *neo*^{R} gene of the N2 vector into mouse hematopoietic cells, followed by successful (48–52%), long-term (up to 200 days) transplantation into irradiated recipients. These results indicate that these packaging lines are safe and efficient for use in experiments designed for murine (using GP+E-86) and human (using GP+*env*Am12) gene therapy.

ACKNOWLEDGMENTS

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Retrovirus Packaging Cells

A. DUSTY MILLER

ABSTRACT

Retroviral vectors promote the efficient transfer of genes into a variety of cell types from many animal species. An important contribution to their utility was the development of retrovirus packaging cells, which allow the production of retroviral vectors in the absence of replication-competent virus. Because of their ability to transfer genes efficiently into cells that are difficult to transfect by other methods, retroviral vectors are prime candidates for gene transfer into human somatic cells. Indeed, a retroviral vector recently has been used to mark tumor infiltrating lymphocytes in patients with melanoma to follow the persistence and distribution of these cells following infusion into patients. Hopefully these vectors will soon be used for the treatment of disease by transfer of functional genes, or gene therapy. Here I will review the available packaging cell lines and their properties with a focus on their ultimate application to human gene therapy.

OVERVIEW SUMMARY

A critical element in the production of the components to carry out retroviral-mediated gene transfer is the cell that generates the retroviral particles carrying the gene to be transferred. The cells are called "packaging cells" because they "package" the retroviral vector (which carries the gene of interest) into a delivery vehicle (the retroviral particles). Miller summarizes our present state-of-knowledge of these "packaging" cells.

RETROVIRUS BIOLOGY

THE STRUCTURE OF THE DNA FORM of a simple replication-competent retrovirus, or helper virus, is shown in Fig. 1. Only one promoter, located in the viral long terminal repeat (LTR), is used to transcribe RNA. Partial splicing of the full-length viral mRNA leads to two RNAs. The full-length mRNA is translated to the *gag* and *gag-pol* polypeptides and the spliced mRNA is translated to the *env* polypeptide. Signals at the ends of the full-length viral RNA direct its

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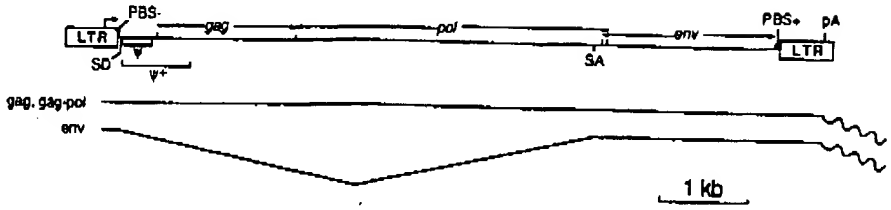

FIG. 1. The structure of the proviral form of a simple retrovirus (MoMLV) and pattern of transcription products. Abbreviations are: PBS−, primer binding site for minus strand DNA synthesis; PBS+, plus strand primer binding site; SD, splice donor; SA, splice acceptor; LTR, long terminal repeat; ψ, retroviral packaging signal (Mann *et al.*, 1983); ψ⁺, extended packaging signal (Bender *et al.*, 1987; Armentano *et al.*, 1987); pA, polyadenylation signal; *gag*, *pol*, and *env*, viral protein coding regions.

encapsidation into virions and subsequent reverse transcription and integration into the genome of an infected cell. Retroviruses can exist in two forms, as free virions that contain viral RNA or as proviruses integrated into cellular DNA.

After the formation of a retrovirus virion, no further viral protein synthesis is required for the events leading to retrovirus integration into the genome of an infected cell. Indeed, all of the viral protein coding regions can be removed from a replication-competent virus without markedly affecting the ability of viral RNA to be encapsidated, reverse-transcribed, and integrated. This is the principle behind the retroviral vector system. The retroviral vector consists of DNA sequences intended for transfer flanked by the signals present at the ends of the retroviral genome, and the packaging cells are designed to produce all of the retroviral proteins and promote "packaging" of retroviral vector RNA into virions. Packaging cells are also called "helper cells" in reference to their ability to "help" a replication-defective virus to replicate, although this terminology may cause confusion between helper cells and helper virus. Retroviral vectors produced by using packaging cells can thus infect cells but cannot replicate further.

The range of cell types from different species that can be infected by a particular retrovirus is called the host range of the virus. There are many complicated viral determinants that influence the host range of a retrovirus (Weiss *et al.*, 1985), the most important of which is the viral envelope protein. Packaging cell lines have been developed based on several retroviruses with different host ranges, including avian leukosis virus, spleen necrosis virus, and ecotropic and amphotropic murine leukemia viruses (Table 1). Of the available packaging cell lines, only packaging cells with an amphotropic host range can produce virus capable of infecting human cells.

PACKAGING CELL CONSTRUCTION

Packaging cell lines are designed to synthesize all retroviral proteins required for assembly of high-titer infectious virus, but should not produce any replication-competent virus. Several reported packaging cell lines meet these criteria and are capable of producing retroviral vectors at 10^6 to over 10^7 infectious units per milliliter of medium exposed to the cells (Table 1). However, packaging cells that are initially helper-free can convert to helper virus-positive during prolonged passage, presumably due to recombination between sequences used to make the packaging cells

TABLE 1. RETROVIRUS PACKAGING CELL LINES

Type[a]	*Name*	*Host range*	*Maximum titer*[b]	*Drug resistance gene(s)*[c]	*Reference*
A	ψ-2	Ecotropic	10^7	*gpt*	Mann *et al.* (1983)
	ψ-AM	Amphotropic	2×10^5	*gpt*	Cone and Mulligan (1984)
	PA12	Amphotropic	4×10^6	*tk*	Miller *et al.* (1985)
	Q4dh	Avian	3×10^5	*hph*	Stoker and Bissell (1988)
B	T19-14X	Amphotropic	10^3	*neo*	Sorge *et al.* (1984)
	VT19-17-H2	Amphotropic	10^3	*gpt*	Sorge *et al.* (1984)
	PA317	Amphotropic	4×10^7	*tk*	Miller and Buttimore (1986)
	PE501	Ecotropic	10^7	*tk*	Miller and Rosman (1989)
	pHF-g	Avian	2×10^4	*hph*	Savatier *et al.* (1989)
C	C3A2	Avian, rat, dog	2×10^7	*neo*	Watanabe and Temin (1983)
	clone 32	Ecotropic	3×10^4	*neo*	Bosselman *et al.* (1987)
D	ψCRE	Ecotropic	10^6	*hph, gpt*	Danos and Mulligan (1988)
	ψCRIP	Amphotropic	10^6	*hph, gpt*	Danos and Mulligan (1988)
E	GP + E-86	Ecotropic	4×10^6	*gpt*	Markowitz *et al.* (1988a)
	GP + *env*Am12	Amphotropic	10^6	*hph, gpt*	Markowitz *et al.* (1988b)
	DSN	Avian, rat, dog	7×10^5	*neo*	Dougherty *et al.* (1989)
	DAN	Amphotropic	4×10^4	*neo*	Dougherty *et al.* (1989)

[a] Packaging cell type based on type of deleted helper virus (Fig. 2).
[b] Highest reported titers. In some cases this value is from papers published after the initial report describing the cell line (*i.e.*, ψ-2, Miller *et al.*, 1988; C3A2, Dornburg and Temin, 1988; PA317, Miller and Rosman, 1989; PE501, A.D. Miller, unpublished results).
[c] Drug resistance gene(s) used to select for DNA transfer during cotransfection of helper virus constructions: *gpt*, xanthine-guanine phosphoribosyltransferase; *tk*, thymidine kinase; *hph*, hygromycin phosphotransferase; *neo*, neomycin phosphotransferase.

and endogenous retrovirus-like elements found in most eukaryotic cells. In addition, introduction of particular retroviral vectors into some packaging cells can result in helper virus production, most likely by recombination between homologous regions of the vector and the defective virus used to make the packaging cells.

Several early packaging cell lines were made by using helper viruses from which the retroviral packaging signal had been deleted (Fig. 2A) (Mann *et al.*, 1983; Cone and Mulligan, 1984; Miller *et al.*, 1985). Although packaging cells of this type have been used with success in a number of studies, most notably the ψ-2 cell line (Mann *et al.*, 1983), these lines suffer from important deficiencies. First, although deletion of the packaging signal reduces the amount of helper virus RNA that is packaged into virions, the block is far from complete. If the deleted helper genome is packaged, there are no further blocks to infection of other cells, because the signals for reverse transcription and integration are intact. Indeed, virus from this type of packaging cells is capable of rescuing retroviral vectors in the absence of overt helper virus production (Cone and Mulligan, 1984; Miller and Buttimore, 1986), presumably due to transfer of the deleted helper virus. Further evidence that deletion of the packaging signal is not sufficient to prevent packaging comes from a study of a retroviral vector without a packaging signal where packaging and transfer of this

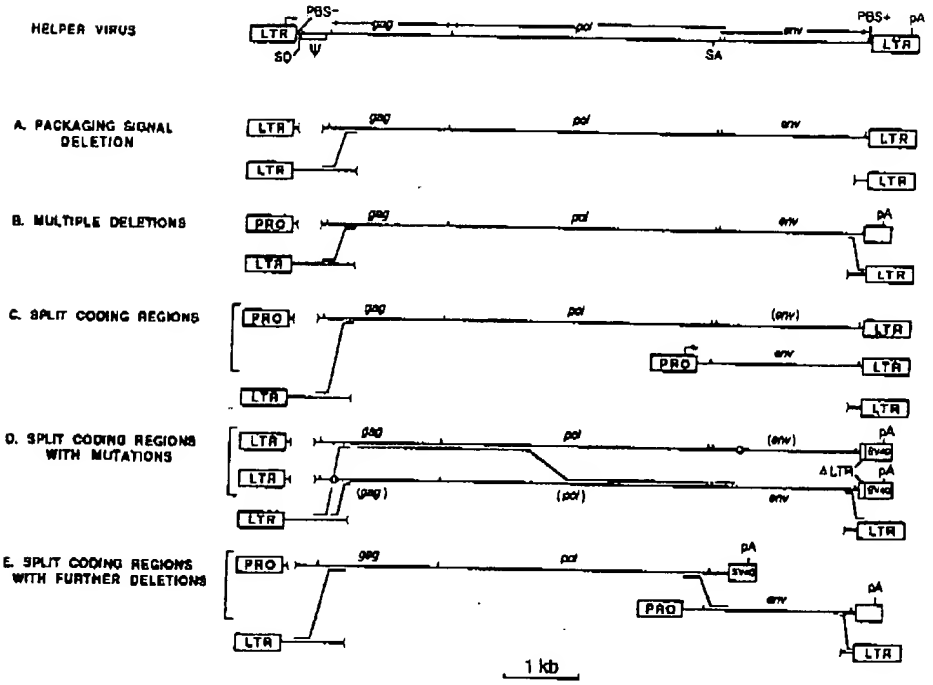

FIG. 2. Strategies for packaging cell line construction. A generic helper virus (modeled on MoMLV) is shown at the top. Strategies used to generate deleted viruses that provide all proteins required for viral replication, but which cannot themselves replicate, are shown. Deleted viruses of types A and B involve single DNA constructs, whereas those of types C, D, and E involve two constructs. The bottom construct in each set shows a generic retroviral vector (or other endogenous retrovirus-like element present in packaging cells). Potential mechanisms for homologous recombination leading to helper virus production are shown between the helper virus and vector constructs, with the extent of homologous overlap and direction of recombination indicated. PRO indicates a promoter, either the viral LTR (Watanabe and Temin, 1983; Sorge *et al.*, 1984; Markowitz *et al.*, 1988a, 1988b; Savatier *et al.*, 1989), the metallothionein promoter (Bosselman *et al.*, 1987), an LTR with a 5′ deletion (Miller and Buttimore, 1986; Miller and Rosman, 1989), or a cytomegalovirus promoter (Dougherty *et al.*, 1989). pA indicates a polyadenylation signal, which where not specified is either that of SV40 (Miller and Buttimore, 1986; Dougherty *et al.*, 1989; Miller and Rosman, 1989), a deleted LTR (Sorge *et al.*, 1984), the polyadenylation signal from the herpes simplex virus *tk* gene (Savatier *et al.*, 1989), or was not explicitly included (Markowitz *et al.*, 1988a, 1988b). Abbreviations in addition to those described in Fig. 1. are: ○, mutation; ΔLTR, deleted LTR.

vector was easily detected, even though the rate was 3,000-fold lower than that of a similar vector containing the packaging signal (Mann and Baltimore, 1985). Thus, deletion of the packaging signal from a helper virus does not completely prevent spread of the virus, although the rate is greatly reduced.

The second problem with packaging cells based on helper viruses with deleted packaging signals is the ease with which the signal can be restored and result in helper virus production (Fig. 2A). Introduction of a retroviral vector that overlaps sequences past the 3′ end of the deletion

reproducibly yields helper virus after short-term passage of the cells (Miller *et al.*, 1986; Miller and Buttimore, 1986; Stoker and Bissell, 1988). In addition, reports of helper virus production in some cell lines after transfection of the deleted helper virus (Mann *et al.*, 1983; Cone and Mulligan, 1984) support the possibility that endogenous sequences in these cells can repair the defect in the helper genome.

Problems encountered with packaging cells made with helper viruses without packaging signals have been reduced by making further deletions in the helper virus genome (Fig. 2B). The best example is the PA317 cell line (Miller and Buttimore, 1986), which allows production of amphotropic retrovirus vectors at high titer. The PA317 cell line was made from a helper virus in which the packaging signal was deleted, the 3′ LTR and second strand initiation site were replaced with a polyadenylation site from SV40, and the 5′ end of the 5′ LTR was deleted. Thus, even if RNA from this construct is packaged it cannot be reverse-transcribed, and sites required for integration of the virus are missing. Indeed, while rescue of a retroviral vector was observed by using virus from packaging lines containing a helper virus without its packaging signal, no rescue was observed with virus from PA317 cells. In addition, PA317 cells containing a vector having sequences that could complement the packaging genome by homologous recombination did not result in helper virus production (Miller and Buttimore, 1986). Two recombination events are necessary to generate helper virus (Fig. 2B), and the frequency of this event is apparently much lower than that of the single event necessary to generate helper from packaging cells containing a helper virus with only the packaging signal deletion (Fig. 2A).

Packaging cells have also been constructed by splitting the *gag-pol* and *env* genes into two seperate transcriptional units. In principle, the advantage of this technique is that an additional recombination event between these elements is required to produce helper virus. However, early packaging lines generated by using this strategy utilized a nearly complete retroviral genome for synthesis of *gag-pol* proteins (Watanabe and Temin, 1983; Bosselman *et al.*, 1987), and thus only one recombinational event between the *gag-pol* transcriptional unit and endogenous retroviral elements or an introduced vector is necessary to generate helper virus (Fig. 2C). Thus, these cell lines are functionally similar to packaging cells made with a helper virus with only a deleted packaging signal, and both packaging cell lines of type C can spontaneously release helper virus during prolonged culture (Bosselman *et al.*, 1987; Hu *et al.*, 1987).

An improvement in packaging cell lines having split coding regions was achieved by making mutations and deletions in the *gag-pol* and *env* transcriptional units (Fig. 2D), as exemplified by the ψCRE and ψCRIP cell lines. In these cell lines, mutations have been made by insertion of linkers (denoted by open circles in Fig. 2D) in either the *gag* or *env* regions of the two transcriptional units. In addition, most of the 3′ LTRs in the separate transcription units have been replaced with polyadenylation signals from SV40. Thus, three recombinational events between the two transcription units and a vector or endogenous element should be required to generate helper virus. However, because of the location of the mutations in the transcription units, only two recombinational events may be necessary to generate helper virus with some retroviral vectors or endogenous retrovirus-like elements (Fig. 2D). In addition, there is extensive overlap between the split coding regions that may increase the chances for a recombination that would repair both mutations (Fig. 2D). There is evidence that the ψCRE and ψCRIP cells are less prone to produce helper virus than PA317 cells when using certain vectors (Danos and Mulligan, 1988), and thus represent an improvement over packaging cells of type B (Fig. 2B).

The most effective application of the split coding region approach involves more extensive deletions in the separate transcriptional units (Fig. 2E). Since the *pol* gene ends after the start of the *env* gene in the parental helper viruses, it is difficult to eliminate overlap between the *gag-pol* and *env* coding regions; thus, helper virus can still be generated by three homologous recombinational events. It is possible to avoid this problem by using a *gag-pol* region from one virus and an *env* region from another virus having little sequence similarity in the region of overlap. This strategy was used in the construction of the DAN amphotropic packaging cell line (Dougherty *et al.*, 1989), where *gag-pol* was derived from spleen necrosis virus and *env* was derived from an amphotropic murine leukemia virus. However, the titer of virus produced DAN cells is very low compared with other amphotropic packaging cells (Table 1).

Another consideration in the choice of a packaging cell line is the presence of particular drug-resistance genes used in cotransfection of the deleted helper virus DNA(s) into the packaging cells (Table 1). For example, vectors carrying the *neo* gene cannot be easily used with packaging cells made by cotransfection with *neo* since the presence of the vector does not confer a selectable phenotype in these cells. Several packaging lines have been made by cotransfection of thymidine kinase (TK)-deficient cells with the *tk* gene; thus, vectors containing any dominant marker can be selected in these cells.

VECTOR DESIGN

The retroviral vector used with a packaging line has a strong influence on the possibility of helper virus production. Avoidance of homologous overlap between vector and helper virus sequences in the packaging cells decreases the chance of helper virus production (Miller *et al.*, 1986; Miller and Rosman, 1989). This can be accomplished by removing as much of the helper virus sequences from the vector as possible. In the case of retroviral vectors based on murine leukemia viruses, overlap at the 5′ end of the vector with helper sequences is difficult to avoid because the packaging signal of these viruses extends into the *gag* region of the helper virus (Armentano *et al.*, 1987; Bender *et al.*, 1987; Adam and Miller, 1988). However, murine leukemia virus-based vectors have been developed that have no *env* sequences, so that overlap between vector and helper sequences at the 3′ end can be eliminated (Miller and Rosman, 1989). Stringent tests for helper virus production by PA317 cells producing an *env*-minus vector were negative (Miller and Rosman, 1989).

Alternatively, areas of homologous overlap between vector and helper virus sequences in packaging cells can be avoided by using a vector derived from a helper virus that has little sequence similarity with the helper virus used to construct the packaging cells. For example, a vector based on spleen focus-forming virus (SFFV) had no tendency to produce helper virus when introduced into murine leukemia virus-based PA12 packaging cells, even though there was extensive overlap between the *gag* regions of the vector and helper DNA. In contrast, a vector based on the same murine leukemia virus as the PA12 cells and having similar overlap as the SFFV-based vector readily produced helper virus in the PA12 cells (Miller *et al.*, 1986). A possible difficulty with this strategy is potential incompatibility between a vector based on a different helper virus than the packaging cells, which might result in low vector titer.

HELPER VIRUS DETECTION

Two types of helper virus production have been observed in packaging cell lines. One type is easy to detect and involves the generation of wild-type virus that can rapidly spread in the packaging cells as well as in cells infected with virus from the packaging cells. The other type is more difficult to detect and involves transfer of packaging function to recipient cells. The virus that is transferred is defective and spreads very slowly in the infected cells. An example of this type of helper virus is a packaging signal-deleted virus (Fig. 2A), which can be packaged and transmitted with low but detectable frequency (Cone and Mulligan, 1984; Miller and Buttimore, 1986).

Several techniques with varying sensitivity are available for the detection of potential helper virus production from packaging cells. The most sensitive are vector rescue assays in which cells containing but not producing a selectable replication-defective retroviral vector are infected with test virus and assayed for production of the vector. Rescue of the vector can be detected by passaging the cells to allow virus spread and assaying medium exposed to these cells for the selectable retroviral vector in a standard colony assay. Alternatively, cells infected with test virus can be cocultivated with recipient cells, which are then assayed for vector infection. An example of the latter assay is a cocultivation assay that measures transfer of a vector carrying the *neo* gene from $HPRT^+$ cells to $HPRT^-$ cells. After cocultivation, HPRT− cells that have been infected with the vector carrying *neo* are measured in a colony assay by using a selection medium that only allows survival of $HPRT^-$ G418-resistant cells (Miller and Buttimore, 1986). Both of these variations of the vector rescue assay can detect overt helper virus production and the more subtle transfer of packaging function.

Events leading to helper virus production can be amplified by passaging virus between packaging cells. Although virus produced by a given packaging cell line does not readily infect cells of the same line due to interference by envelope protein made by the packaging cells (Miller *et al.*, 1986), virus can be passaged between different lines to avoid this interference. For example, retroviral vectors have been cycled between ecotropic and amphotropic packaging cells and then assayed for helper virus production in a vector rescue assay (Danos and Mulligan, 1988; Miller and Rosman, 1989). This type of assay is currently the most stringent method available for assessing potential helper virus from packaging cells.

GENE TRANSFER INTO HUMANS

Of the retrovirus packaging cell lines developed to date, only vectors produced by amphotropic packaging cells will infect human cells. Because the possibility of helper virus production by any of the available amphotropic packaging cells can never be excluded entirely, it was necessary to address this issue before using retroviral vectors in humans. Several arguments suggest that potential helper virus production is of minor concern. First, human complement is known to inactivate murine retroviruses, including the amphotropic variety, by direct lysis of virions in an antibody-independent manner (Welsh *et al.*, 1975; Cooper *et al.*, 1976; Welsh *et al.*, 1976). This is in contrast to the inability of human serum to inactivate retroviruses that cause disease in humans, human T-lymphotropic virus-I (HTLV-1) (Hoshino *et al.*, 1984) and human immuno-

deficiency virus (HIV) (Banapour *et al.*, 1986). The site of complement action is the viral envelope protein (Bartholomew *et al.*, 1978). Recombinant viruses between Moloney murine leukemia virus (MoMLV) and amphotropic virus 4070A, upon which most amphotropic packaging cell lines are based, are also sensitive to complement inactivation (Cornetta *et al.*, 1990; R.A. Hock and A.D. Miller, unpublished results). This activity should reduce the possibility of viral infection or virus spread *in vivo*. Second, the possibility of helper virus production by amphotropic packaging cells of types B, D, and E is very low when using an appropriate vector, as measured by very sensitive assays (Danos and Mulligan, 1988; Markowitz *et al.*, 1988b; Miller and Rosman, 1989).

PA317 amphotropic packaging cells producing a retroviral vector carrying the *neo* gene (PA317/LNL6 cells, Bender *et al.*, 1987) have recently been used to transduce human tumor infiltrating lymphocytes (TIL cells) that were subsequently infused into patients with advanced melanoma. The procedure was performed to follow the distribution and persistence of the cells in the patient, especially at tumor sites where the TIL cells can mediate tumor cell killing. Prior to use of the packaging cells, they were shown to be free of standard cell culture contaminants, including bacteria, fungi and agar cultivable and noncultivable mycoplasmas. The packaging cells were also free of other more exotic contaminants, including 15 viruses that can infect mice, adventitious agents visible by electron microscopy or by their ability to induce cytopathology in indicator cells, and ecotropic, xenotropic or amphotropic replication-competent murine retroviruses. Many liters of virus have been harvested from PA317/LNL6 cells without the appearance of helper virus. Initial results in humans have shown no ill effects from the use of the virus (see News and Comments, this issue). These results are encouraging and hopefully anticipate the more widespread use of these vectors for gene transfer and gene therapy in humans.

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Biochemistry

Cells that express all five proteins of vesicular stomatitis virus from cloned cDNAs support replication, assembly, and budding of defective interfering particles

(vaccinia virus-T7 RNA polymerase/coexpression/helper-independent budding)

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ABSTRACT **An alternative approach to structure–function analysis of vesicular stomatitis virus (VSV) gene products and their interactions with one another during each phase of the viral life cycle is described. We showed previously by using the vaccinia virus-T7 RNA polymerase expression system that when cells expressing the nucleocapsid protein (N), the phosphoprotein (NS), and the large polymerase protein (L) of VSV were superinfected with defective interfering (DI) particles, rapid and efficient replication and amplification of DI particle RNA occurred. Here, we demonstrate that all five VSV proteins can be expressed simultaneously when cells are cotransfected with plasmids containing the matrix protein (M) gene and the glycoprotein (G) gene of VSV in addition to plasmids containing the genes for the N, NS, and L proteins. When cells coexpressing all five VSV proteins were superinfected with DI particles, which because of their defectiveness are unable to express any viral proteins or to replicate, DI particle replication, assembly, and budding were observed and infectious DI particles were released into the culture fluids. Omission of either the M or G protein expression resulted in no DI particle budding. The vector-supported DI particles were similar in size and morphology to the authentic DI particles generated from cells coinfected with DI particles and helper VSV and their infectivity could be blocked by anti-VSV or anti-G antiserum. The successful replication, assembly, and budding of DI particles from cells expressing all five VSV proteins from cloned cDNAs provide a powerful approach for detailed structure–function analysis of the VSV gene products in each step of the replicative cycle of the virus.**

A detailed analysis of the structure and function of negative-strand RNA virus proteins and their interactions with one another in the replicative cycle has suffered from the lack of a method for genetic manipulation. Introduction of site-directed alterations in genomes of negative-stranded RNA viruses has been limited because the naked genomic RNA is not biologically active or infectious. The functional template for transcription and replication is the nucleocapsid structure that contains the genomic RNA encapsidated with nucleocapsid protein. Several different approaches are now available to generate viral genomic RNAs from cDNA clones *in vitro*. However, for negative-strand viruses, these genomic RNAs must be assembled into nucleocapsid structures to obtain biological activity. The difficulty in generating biologically active nucleocapsids of negative-strand RNA viruses has provided a major stumbling block for detailed genetic analysis of this group of viruses. Only recently, synthetic transcripts representing the genomic RNAs of two negative-strand RNA viruses, measles and influenza, have been assembled into biologically active nucleocapsids (1–3). These accomplishments hold great promise as experimental systems for genetic manipulation of negative-strand virus genomes. In both cases, however, the success of the system was closely tied to the biology of the virus. Therefore, alternative approaches to study structure–function analysis of viral proteins would be useful.

Vesicular stomatitis virus (VSV), a rhabdovirus, contains five structural proteins and each of these proteins plays a role in replication, assembly, and budding of VSV. The genomic RNA of VSV is a single-stranded 11,161-nucleotide-long RNA (4, 5) of negative polarity, which is encapsidated with the nucleocapsid protein (N) as a nucleocapsid structure. The active template for transcription and replication is the nucleocapsid structure that contains two other minor proteins, the phosphoprotein (NS) and the large polymerase protein (L), both of which are required for the RNA polymerase activity of VSV (6–8). These three proteins—namely, the N, NS, and L proteins—are the only proteins that are required for transcription and replication of VSV (8). The ectodomain of the glycoprotein (G) of VSV forms the spikes on the viral envelope and interacts with virus receptors on susceptible cells (9). There is evidence that the cytoplasmic domain of the G protein is required for assembly of VSV (10, 11). Several lines of evidence indicate that the matrix protein (M) plays a crucial role in the assembly and budding processes of VSV. The M protein assembles at the inner surface of the plasma membrane and is thought to interact with the G protein and the viral nucleocapsid structures (12–15). The M protein is also involved in the condensation of nucleocapsids into the tightly coiled structures that are found in mature virions (16, 17).

In this communication, we describe an alternative approach to the study of VSV proteins and their interactions with each other during replication, assembly, and budding of VSV. We have previously shown that transfection of plasmid DNAs containing VSV genes under the control of a T7 RNA polymerase promoter into cells infected with a recombinant vaccinia virus that contains and expresses the gene for bacteriophage T7 RNA polymerase results in high-level expression of the VSV proteins (8). Coexpression of the N, NS, and L proteins of VSV in cells allows replication and amplification of defective interfering (DI) particle RNA of VSV (8). We and others have also shown that the G and M proteins can be expressed in functional forms by using the same expression system (11, 18–20). We demonstrate here that by transfecting plasmids containing cDNA clones for all five of the VSV genes into cells, it is possible to express all five VSV proteins such that they can support replication,

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Abbreviations: VSV, vesicular stomatitis virus; DI, defective interfering; N protein, nucleocapsid protein; NS protein, phosphoprotein; L protein, large polymerase protein; M protein, matrix protein; G protein, glycoprotein.

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assembly, and budding of infectious DI particles from these cells. This system will allow us to study detailed structure–function aspects of each of the viral proteins and the role of these proteins in each step of the viral replicative cycle.

MATERIALS AND METHODS

Cell Cultures and Viruses. Baby hamster kidney cells (BHK-21) were maintained as monolayer cultures in minimal essential medium (MEM) containing 5% heat-inactivated fetal bovine serum and 5% newborn calf serum. VSV (Indiana serotype, San Juan strain) was propagated in BHK-21 cells. Stocks of DI-T particles (21) were prepared as described (8). Recombinant vaccinia virus (vTF7-3) containing the T7 RNA polymerase gene has been described (22) and was kindly provided by Bernard Moss (National Institutes of Health, Bethesda, MD). Stocks of vTF7-3 were prepared and infectivity titers were determined as described (8).

Plasmid Vectors and cDNA Clones. The plasmids pAP-N, pMB-NS, and pAP-L containing the N gene, the NS gene, and the L gene, respectively, have been described (8). The plasmid pKOM2 containing the wild-type M gene (Indiana serotype) inserted into the pGEM4XB vector has been described (23) and was kindly provided by Manfred Schubert (National Institutes of Health). This plasmid contains the M gene under the control of T7 RNA polymerase promoter. The plasmid pTF-G contains the VSV G gene (Indiana serotype) under the control of the T7 RNA polymerase promoter and transcriptional terminator in pTF7-5 (24). This plasmid was kindly provided by M. Abdul Jabbar (University of California-Los Angeles, Los Angeles). Plasmids were prepared and purified as described (25).

Virus Infection, DNA Transfection, and Radioactive Labeling. BHK-21 cells in 60-mm plates were infected with the recombinant vaccinia virus vTF7-3 and transfected with plasmid DNA using the calcium phosphate precipitation method as described (8, 26). Radioactive labeling of proteins with [^{35}S]methionine (20 μCi/ml; 1 Ci = 37 GBq) and of RNA with [^{3}H]uridine (20 μCi/ml) was also performed as detailed (8).

Immunoprecipitation and Electrophoretic Analysis of Proteins. Cytoplasmic extracts of cells were prepared and virus-specific proteins were immunoprecipitated as described (8) by using either a mouse polyclonal antiserum raised against purified VSV or a monospecific rabbit antibody raised against an amino-terminal peptide from the L protein (27) kindly supplied by Manfred Schubert or both. Proteins were analyzed by electrophoresis in 10% polyacrylamide gels by using the buffer system of Laemmli (28) and detected by fluorography (29).

Analysis of DI Particle RNA Replication. Replication of DI particle RNA was analyzed by immunoprecipitation of nucleocapsids as described (8). [^{3}H]Uridine-labeled RNAs recovered from immunoprecipitated nucleocapsids were resolved by electrophoresis in 1.75% agarose/citrate/urea gels (30) and detected by fluorography (31).

Electron Microscopy. DI particles generated from cells coinfected with helper VSV and DI particles or from cells expressing all five VSV proteins were analyzed by transmission electron microscopy. Clarified culture fluids were pelleted through a cushion of 5% sucrose in 10 mM Tris·HCl, pH 7.4/100 mM NaCl/1 mM EDTA (NTE). The pellets were resuspended in NTE, and aliquots of each sample were negatively stained with uranyl acetate and visualized by using a Phillips 301 transmission electron microscope.

Antibodies. Anti-VSV antibodies were raised in mice against gradient-purified VSV. The monoclonal antibody (DC3) to the G protein was kindly provided by Richard Compans (University of Alabama at Birmingham). The monoclonal antibodies (10G4, 22F9, 19B10, and 8C11F10) to the VSV N protein were a generous gift from D. Lyles (Wake Forest University, Winston-Salem, NC).

RESULTS

Expression of Viral Proteins in Transfected Cells. We have previously shown (8) that the N protein, the NS protein, and the L protein of VSV can be coexpressed in HEp-2 cells by cotransfection of plasmids containing the individual VSV genes using the recombinant vaccinia virus-T7 RNA polymerase expression system (22). The N, NS, and L proteins expressed in this manner were shown to support replication of DI particle RNA (8). Here we determined whether all five VSV proteins could be coexpressed in cells by cotransfection of plasmids containing the genes for each of the five VSV proteins. BHK-21 cells cotransfected with plasmids containing the individual VSV genes were labeled with [^{35}S]methionine. Total cytoplasmic proteins or immunoprecipitated proteins from cytoplasmic extracts were analyzed by SDS/PAGE. All five VSV proteins were coexpressed in cultures cotransfected with all five plasmids (Fig. 1, lanes 4 and 7). The molar ratios of the G, N, and M proteins expressed in cotransfected cells and that of the proteins present in VSV-infected cells were comparable (lanes 7 and 8). Significant levels of the L and NS proteins were expressed in cotransfected cells (lanes 3, 4, 6, and 7). However, the amounts of the L and NS proteins relative to other VSV proteins in cells cotransfected with all five plasmids were less than that in VSV-infected cells (compare lanes 4, 7, and 8) or that in cells expressing only the N, NS, and L proteins (lanes 3 and 6). Further experiments have demonstrated that the expression of the M protein depresses the level of expression of other VSV proteins when coexpressed (unpublished results).

Assembly and Budding of DI Particles from Cells Expressing All Five VSV Proteins. The ability of the five VSV proteins coexpressed in cells by transfection of plasmids containing each of the five VSV genes to support DI particle RNA

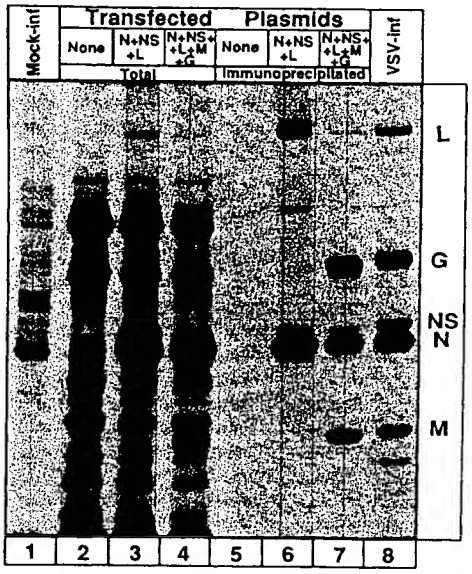

FIG. 1. Coexpression of VSV proteins in cotransfected cells. Cells infected with vTF7-3 and transfected with no plasmid (lanes 2 and 5), 5 μg of pAP-L, 10 μg of pMB-NS, and 15 μg of pAP-N (lanes 3 and 6), or 5 μg of pAP-L, 10 μg of pMB-NS, 15 μg of pAP-N, 10 μg of pKOM2, and 15 μg of pTF-G (lanes 4 and 7) were labeled with [^{35}S]methionine. Total cytoplasmic proteins (lanes 2–4) or proteins immunoprecipitated from cytoplasmic extracts with anti-VSV antibodies (lanes 5–7) were analyzed by SDS/PAGE. Proteins from mock-infected (lane 1) and VSV-infected (lane 8) cells are also shown.

replication, assembly, and budding was examined. The DI particles cannot replicate on their own because they do not contain the genetic information to code for any functional VSV proteins. Normally, coinfection with helper virus is required to provide the functional viral proteins for replication, assembly, and budding of the DI particles. The protocol to analyze assembly and budding of DI particles from cells expressing viral proteins is as follows. Cells infected with vTF7-3 were transfected with various combinations of plasmid DNAs containing the individual genes for the VSV proteins. At 5 hr after transfection, the cells were superinfected with DI particles. After virus adsorption, the cells were washed twice in warm MEM and then incubated at 37°C with 1 ml of MEM. Culture fluids from these cells (passage 1) were collected at 20–24 hr after DI particle superinfection and clarified by centrifugation at 16,000 × *g* for 1 min at 4°C. Half (500 μl) of the clarified culture fluids was used to infect (as in passage 1) a second set of cells (passage 2) that had been infected with vTF7-3 and transfected with the same combination of plasmid DNAs as in the first passage. Culture fluids from passage 2 were collected at 20–24 hr after infection and clarified as above. It was necessary to carry out two passages to avoid the possible carry-over of any DI particles used in the primary infection that may have been present in passage 1 culture fluids due to the release of adsorbed DI particles that did not penetrate the cells. The presence of infectious DI particles in the culture fluids from passage 2 cells was assayed by the RNA replication assay (8) in cells expressing the N, NS, and L proteins of VSV. After two passages, DI particles were not detected in the culture fluids of cells that did not express any viral proteins (Fig. 2, lane 2) or cells that expressed only the N, NS, and L proteins (lane 3). Expression of the N, NS, and L proteins in combination with either the M or G proteins also did not support viral budding (Fig. 2, lanes 4 and 5, respectively). DI particles were detected, however, in the culture fluids of cells that expressed the M and G proteins in addition to the N, NS, and L proteins (lane 6). These results show that DI particle budding occurred only when all five VSV proteins were coexpressed and that the M and G proteins are required for assembly and budding of infectious DI particles.

To demonstrate that the vector-supported budded DI particles were mature particles containing the viral G protein in the envelope with the nucleocapsids enclosed inside, we tested antibodies against the G or N protein or against all five VSV proteins for their ability to block the infectivity of the vector-supported budded particles present in the culture fluids. Purified authentic DI particles or the budded particles from culture fluids of cells expressing all five VSV proteins were treated separately with antibodies raised against VSV, a monoclonal antibody to the G protein, or a combination of four monoclonal antibodies to the N protein. The monoclonal antibody to the G protein neutralizes the infectivity of VSV (unpublished result). The monoclonal antibodies to the N protein are known to bind nucleocapsids (J. Glass and G.W.W., unpublished observation) and previous work has shown that such anti-N monoclonal antibodies block the transcription and replication capabilities of nucleocapsids (32). The ability of antibody-treated DI particles and vector-supported budded particles in the culture fluids to direct replication of their genomic RNA was examined in cells that were infected with helper VSV. The results in Fig. 3 show that anti-VSV and anti-G antibodies inhibited the genomic RNA replication of authentic DI particles used as a control (lanes 2 and 3) as well as that of the DI particles budded into the culture fluids of cells expressing all five VSV proteins (lanes 6 and 7). However, the anti-N antibodies (lanes 4 and 8) had no effect on infectivity, and RNA replication directed by both types of DI particles was observed at levels similar to those obtained in the absence of the antibodies (lanes 1 and 5). Taken

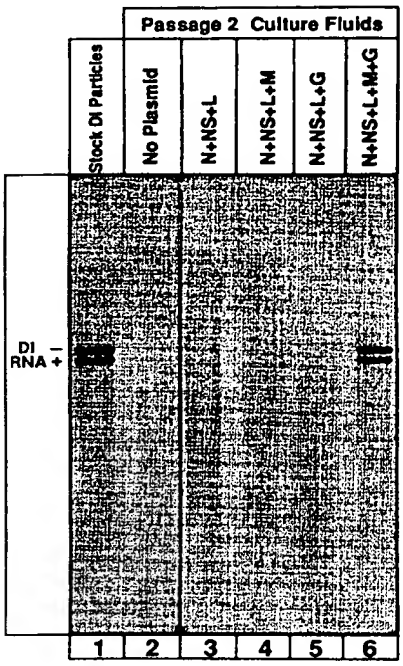

FIG. 2. Assay for the presence of DI particles in the culture fluids. Passage 2 culture fluids from cells transfected with no plasmid (lane 2), 15 μg of pAP-N, 10 μg of pMB-NS, and 5 μg of pAP-L (lane 3), 15 μg of pAP-N, 10 μg of pMB-NS, 5 μg of pAP-L, and 10 μg of pKOM2 (lane 4), 15 μg of pAP-N, 10 μg of pMB-NS, 5 μg of pAP-L, and 10 μg of pTF-G (lane 5), or 15 μg of pAP-N, 10 μg of pMB-NS, 5 μg of pAP-L, 10 μg of pKOM2, and 10 μg of pTF-G (lane 6) were analyzed for DI particles using an RNA replication assay. The RNA replication assay was performed by labeling with [^{3}H]uridine in cells that were transfected with 15 μg of pAP-N, 10 μg of pMB-NS, and 5 μg of pAP-L plasmid DNA. Labeled RNAs present in immunoprecipitated nucleocapsids from the cytoplasmic extracts were analyzed in an agarose/urea gel. Lane 1 shows DI RNA replication supported by the same system using 500 μl of 1:500 dilution of authentic purified DI stock virus.

together, these results indicate that the particles that are present in the culture fluids of cells expressing all five VSV proteins contained G protein on the surface of the envelope and that the nucleocapsid structures were enclosed within the envelope in a manner similar to that of the authentic DI particles generated from cells coinfected with helper VSV.

The presence of budded DI particles in culture fluids was also examined by electron microscopy. The culture fluids of cells coinfected with helper VSV and DI particles contained a large number of DI particles in addition to some VSV particles (Fig. 4*A*). These DI particles were approximately one-third of the size of the typical bullet-shaped wild-type VSV particles and appeared to be spherical. Particles similar in size and morphology to the authentic DI particles were also seen in the culture fluids of cells expressing all five VSV proteins (arrows in Fig. 4*B*). Culture fluids of vTF7-3-infected cells expressing only the N, NS, and L proteins did not contain any particles similar to authentic DI particles (data not shown). The number of particles generated from cells expressing all five VSV proteins amounted to 5–10% of the DI particles generated from helper VSV-coinfected cells.

DISCUSSION

In this report, we demonstrate that cells expressing all five structural proteins of VSV from cloned cDNAs, when infected with DI particles, allow replication, assembly, and

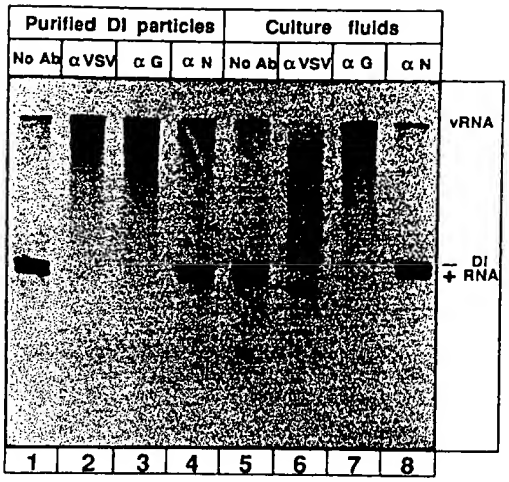

FIG. 3. Infectivity of DI particles in the presence of various antibodies. Purified DI stock virus or DI particles present in passage 2 culture fluids from cells transfected with amounts of plasmid DNA as described in Fig. 2, lane 6, were treated with no antibodies (lanes 1 and 5), anti-VSV antibody (1:25 dilution; lanes 2 and 6), monoclonal antibody DC3, to the G protein (1:25 dilution; lanes 3 and 7), or a combination of four monoclonal antibodies to N protein (1:50 dilution of each; lanes 4 and 8) for 2 hr at 4°C. The DI stock virus or culture fluids were then used in the RNA replication assay in cells infected with helper VSV as described (8).

budding of infectious DI particles. Expression of all five VSV proteins is absolutely required for DI particle assembly and budding since expression of the N, NS, L, and either the M or G proteins did not support particle budding. The infectivity of these particles could be neutralized by anti-VSV and anti-G antisera but not by anti-N antiserum, indicating that these particles contained G protein on the envelope with the nucleocapsid enclosed inside. The particles were also similar in size and morphology to the authentic DI particles generated from cells coinfected with helper VSV.

Cotransfection with plasmid DNAs containing all five VSV genes resulted in synthesis of all five VSV proteins. The molar ratios of the G, N, and M proteins in cells coexpressing all five VSV proteins were comparable to those of the cells infected with VSV. The NS and L proteins, however, were synthesized in amounts that were less than those seen in VSV-infected cells. In previous work, we showed that in cells cotransfected with plasmids bearing the genes for the N, NS, and L proteins, expression of one protein did not interfere with the expression of other VSV proteins (8). This, however, was not found to be the case in cells coexpressing all five VSV proteins. Coexpression of M protein with other VSV proteins led to inhibition of expression of other VSV proteins. This result is intriguing, particularly in the context of the vaccinia virus T7-RNA polymerase expression system, since the M protein has been shown to inhibit VSV RNA transcription by condensing the nucleocapsids into transcriptionally inactive forms (33–35). In VSV-infected cells, VSV-specific proteins are synthesized by polyribosomes in association with the cytoskeletal framework (36) and it has been postulated that the M protein may disrupt the cytoskeletal framework (23). It is possible, therefore, that the M protein inhibits the expression of other VSV proteins at the level of translation. Further experiments are required to examine the mechanism of inhibition of protein expression by the M protein.

We have shown previously that the level of proteins in transfected cells can be controlled by the amount of DNA plasmid transfected into the cell. Therefore, it should be

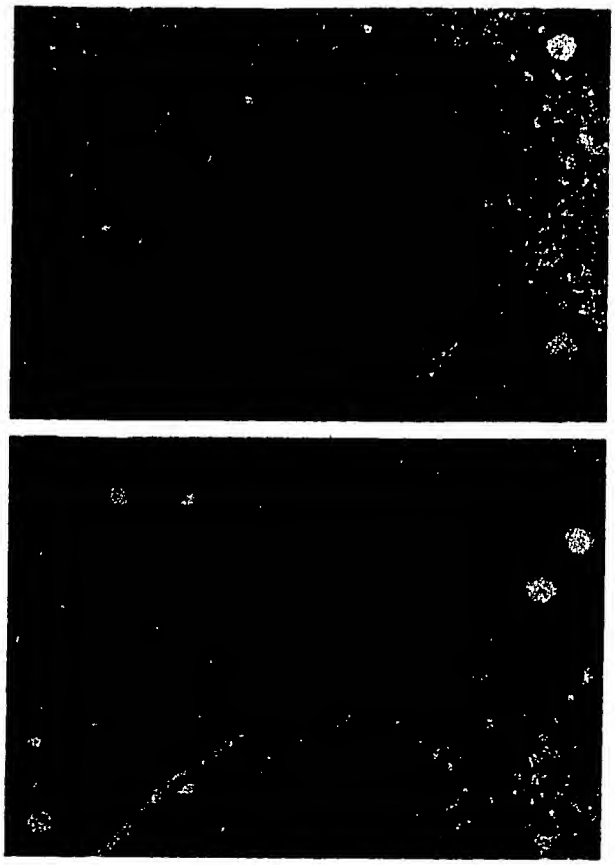

FIG. 4. Electron microscopic analysis of DI particles. DI particles isolated and partially purified from culture fluids of cells coinfected with helper VSV and DI particles (*A*) or from cells transfected with all five plasmids as described in Fig. 3, lane 6, and infected with DI particles (*B*) are shown. Arrows in *B* show the vector-supported budded particles. (Bar = 200 nm.)

possible to increase the expression of the NS and L proteins by increasing the amount of the corresponding plasmid DNAs in transfection such that the molar ratios of all five VSV proteins are comparable to those seen in VSV-infected cells. Nevertheless, the levels of proteins synthesized in cells transfected with all five plasmids under the conditions used here supported replication, assembly, and budding of DI particles. DI particle budding from cells expressing all five VSV proteins was readily detectable using the RNA replication assay and the number of budded particles represented 5–10% of those obtained from helper VSV-coinfected cells. Using HEp-2 cells, we have shown previously that the amount of DI particle RNA replication in cells expressing the N, NS, and L proteins is 8–10 times more than that in cells infected with VSV (8). However, in BHK-21 cells (used here because they are stable to long-term vaccinia virus infection) the amount of DI particle RNA replication in cells expressing the three VSV proteins is less than that in cells infected with VSV (unpublished observation). The reduced level of vector-supported budding of DI particles may be a result of the reduced level of RNA replication in BHK-21 cells or due to synthesis of suboptimal levels of the five VSV proteins or both. The fraction of the cells that express all five VSV proteins has not been determined yet but this would influence the number of cells that would support DI particle budding. We determined previously that ≈40% of the cells in culture

coexpressed the N, NS, and L proteins when cotransfected with the corresponding plasmid DNAs (8).

Using the vaccinia virus-T7 RNA polymerase expression system, Li *et al.* (19, 20) were able to express the M protein that could complement a temperature-sensitive mutant (tsO23) with a defect in the M gene. However, Blondel *et al.* (23) were unable to detect stable expression of the M protein by using a simian virus 40 expression system. The inability to detect the M protein expression was correlated with rounding and rapid loss of cells expressing the M protein. When the M protein was expressed alone or with other VSV proteins, we did not observe any cell rounding or detachment even at 20 hr after transfection in the experiments described here. It is hypothesized that the ability of M protein to cause cell rounding and subsequent detachment of cells is antagonized in vaccinia virus-infected cells.

The ability to generate infectious DI particles from cells expressing all five VSV proteins provides a way to apply reverse genetics to VSV. In particular, detailed structure–function analysis of VSV proteins and their interactions with each other during RNA replication, assembly, and budding of virus particles can now be undertaken. The replication system has been used already to analyze the functional defects in temperature-sensitive mutants of VSV with lesions in the N gene (ref. 37; J. Glass and G.W.W., unpublished observations). Additionally, dominant negative deletion mutants of NS protein that interfere with the normal functioning of the wild-type NS protein have been identified (ref. 38; M. Howard, A.K.P., and G.W.W., unpublished observations). Studies on the assembly and budding of DI particles containing heterologous glycoproteins or chimeras between VSV G protein and other viral glycoproteins should provide information on the mechanisms of generation of viral pseudotypes. Furthermore, in VSV-infected cells positive- and negative-strand RNAs are encapsidated but only the nucleocapsids containing the negative-strand RNA are incorporated into budded virions. The mechanism by which this selection is exerted is not known at present but can be addressed by experiments using the system we have described above. Most importantly, the system we have described above provides the ability to make specific changes in each of the individual proteins of VSV and to study the role of the viral proteins in each step of the replicative cycle of VSV—that is, replication, assembly, and budding. In addition, it is anticipated that this system should be applicable to other negative-strand virus systems for which complete cDNA copies of the viral genes are available.

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